Textbook of MICROBIOLOGY

BURROWS

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Textbook of MICROBIOLOGY

Eighteenth Edition

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Preface

In the more than quarter of a century during which the author has been responsible for this book, the immense vitality of microbiology has been apparent in many ways. By far the most important is its continuously increasing importance as a basic biological science through unique contributions to basic biological concepts in contrast to growth through simple accretion of information not hitherto available. In fact, it is now not only possible, but practical and profitable as well, to make use of a literal analysis of the term "microorganism" as a foundation for the discipline.

In keeping with this, the integrated treatment of bacteria, rickettsiae, and viruses with respect to morphology, growth, and genetics on the common ground of nucleic acid replication, which was initiated in the immediately preceding edition, has been continued and amplified. This amplification is reflected in many ways, such as expansion of the sections on the replication of animal viruses, complete rewriting of those concerned with pentose metabolism and the synthesis of nucleic acid and of protein, and the inclusion of information not previously available in new sections on interferon, infectious nucleic acid, etc.

A new section on the use of the embryonated hen's egg and of continuous cell line and primary cell cultures has been added to the chapter on laboratory methods in view of the general introduction of the techniques of virus culture into basic microbiological training. Similarly, a new section on fluorescent antibody staining reflects the now almost routine application of this powerful tool. Other new material is included in various sections, such as those on lipid metabolism of bacteria, the permeases, the anonymous mycobacteria, and others. Much other material has been largely or completely rewritten. Thus the remarkable progress in studies on the virulence of the plague bacillus, and the consolidation of intensive investigation of the past decade on the epidemiology of plague, have required extensive rewriting of these aspects of Pasteurella. It has been necessary, too, to rewrite completely the section on Listeria, and the consideration of antibiotics to include the fungal antibiotics and newer penicillins as well as the now possible chemical classification of these agents has been appropriately rewritten. Similarly, the chapters on staphylococci and streptococci, and sections such as those on trachoma, endotoxins, and on immunization to anthrax and poliomyelitis have also been rewritten in large part.

The problems of nomenclature assume great significance in a book such as this since nomenclature represents the common coinage of communication. These matters have required, in the opinion of the author, a conservative approach to avoid to a degree facile alterations of

generic and specific names, and to allow the student to read the literature of other than his own nationality or decade with a minimum of the confusion inherent in a constantly shifting nomenclature. It is, therefore, a source of considerable personal satisfaction to see the timely resolution of the problems of nomenclature evolving through the efforts of the International Association of Microbiological Societies. With substantially complete solution of such questions, the onus of decision can be removed from editors and authors to the great advantage of the student. Until this state of affairs is more fully realized, it will continue to be necessary to equate systems of nomenclature in some areas, as among certain of the enteric bacilli.

One of the more overtly apparent changes is the result of a decision to document the text with complete references ordered at the ends of the chapters rather than with the multiple abbreviated footnotes used heretofore. The system of using superscript numbers in the text to refer to appropriate publications arranged in alphabetical order is the most time-consuming and rigid form for an author, but has been adopted here to avoid complication of the text with a multiplicity of names and at the same time make possible the convenience of an alphabetized bibliography.

The references are given in full, with inclusive pages and titles in the original language. Their selection continues to be based, as noted in an earlier (13th) edition, on the needs of the student, with consequent emphasis on reviews, symposia, and similar compilations leading further into the relevant literature; the occasional inclusion of case reports is not anomalous for these are selected because they include extensive bibliographies. The great majority of references are to papers written in English as the original language or, as in the case of certain Russian papers, to English translations of the complete report. At the same time, there has been no hesitation in including a minimal number of reports written in French, German, and occasionally Spanish, which contribute uniquely to the coverage. For example, the most extensive recent review of tetanus immunization, including more than a thousand references given in full, is that of Regamey which is written in French and published in a German language journal. Similarly, the significant studies of Mme. Staub and her associates on the chemical structure of Salmonella antigens are available only in French, and the reports making up a summation of the present status of the epidemiology of plague are written largely in French, and the classic writings of Bustamante are in Spanish. While bibliographic compilations are inevitably individual in nature, the author believes that those given here will effectively facilitate introduction to the extensive literature of microbiology.

With this edition Dr. Robert M. Lewert has assumed responsibility for the chapter on medical parasitology, and Dr. J. W. Moulder has continued to carry the chapter on bacterial metabolism. Dr. Dorothy Hamre has prepared the sections on egg and tissue culture, and has been extremely helpful in many other ways. The author is also greatly indebted to very many of his colleagues for advice and criticism, and for material which would not have been otherwise available. Special thanks are expressed to Drs. B. A. Freeman, W. R. Martin, C. F. Simpson, F. H. White, R. S. Benham, and R. R. Brubaker. Miss Sabina Wagner and Mrs. Eve Kyger have given invaluable assistance in much of the bibliographic work.

William Burrows

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Chapter 1

THE HISTORICAL DEVELOPMENT OF MICROBIOLOGY

THE TERM microbiology is commonly used in a more narrow sense than its etymology suggests. It does not refer to the broader subject of small or minute living organisms, but rather has come to be restricted to those microorganisms that are directly or closely related to human activity and welfare.

The microorganisms as a group resist precise taxonomic limitation in that they spread over both the plant and animal kingdoms, from the fungi of obvious plant affinities to the unicellular and small multicellular animals. In this scheme of things the true bacteria, or Eubacteriales, occupy a position between the two kingdoms and in a real sense constitute a link between them. The viruses, distinguished by their obligate parasitic relationship to the cells of their hosts, are perhaps to be regarded as an offshoot of the bacteria, and are of unique interest in that many of them seem to occupy a borderline position between the living and the nonliving.

The consequences of the varied activities of many of the microorganisms have been familiar to man from prehistoric times. Decomposition of organic matter and especially spoilage of foods, acetic and lactic acid fermentations and alcoholic fermentation, degradation of proteins with the production of new and desirable flavors in certain foods, and the occurrence of infectious disease are obvious examples of familiar phenomena now known to be of microbial etiology.

The existence of these etiological agents may be inferred from such consequences of their activity, and their living nature from the ability to reproduce the observed effects indefinitely in series by, for example, the transfer of a small portion of a fermenting mixture to fresh, unfermented substrate. It is probable that such inferences were made early, for many statements may be found in both ancient and later writings which may be interpreted as supporting this idea. Thus Lucretius writes of "the seeds of disease" in his *De Rerum Natura,* Fracastorius of Verona in 1546 suggested a *contagium vivum* as a cause of disease, and von Plenciz accounted for the specificity of disease on the basis of a microbial etiology in 1762.

Nevertheless, appreciation of the abstract concepts commonplace in modern science is a relatively recent development, and even yet these are often accepted by man in general more on faith than on intellectual understanding. So an essential feature of the beginnings of a scientific microbiology was the demonstration of these agents in such a way that they were more or less directly evident to the senses. This was accom-

plished by the development of optical systems of sufficient precision to allow them to be seen.

MICROSCOPY

The name of Antony van Leeuwenhoek is inseparably associated with the early development of microscopy. Van Leeuwenhoek lived in the latter part of the seventeenth and early part of the eighteenth centuries (1632–1723) in Delft. He held a political sinecure and was able to devote the greater part of his time to the hobby of lens grinding. He not only made the best lenses available to that time, but used them in the examination of a wide variety of materials that interested him. It was characteristic that he first observed bacteria in an attempt to discern in visual terms the nature of the taste of pepper. There is no doubt that he actually observed these microorganisms for he made recognizable drawings of them that were reproduced in his report[7] of his observations to the Royal Society. In the relevant portion of this report he wrote:

> Having several times endeavoured to discover the cause of the pungency of pepper upon our tongue, and the rather because it hath been found, that though pepper had layn a whole year in vinegar, yet it retained still its pungency; I did put about ⅛ of an ounce of whole pepper in water, placing it in my study, with this design, that the pepper thereby being rendered soft, I might be enabled the better to observe what I had proposed to myself. This pepper having lain about three weeks in the water, to which I had added some snow water, the other water being in great part exhaled, I looked upon it the 24th of April, 1676 and discern'd in it, to my great wonder, an incredible number of little animals, of divers kinds. . . . The 4th sort of creatures, which moved through the three former sorts, were incredibly small and so small in my eye, that I judge, that if 100 of them lay one by another, they would not equal the length of a grain of coarse sand; and according to this estimate, ten hundred thousand of them could not equal the dimensions of a grain of such sand.

A few of the hundreds of microscopes he constructed are still available, and it is evident that the maximum magnification he reached was approximately 300 diameters. This is not sufficient to allow observation of objects as small as bacteria by transmitted light. While van Leeuwenhoek was never willing to reveal his method of illumination, it seems probable that he used reflected light as it is used in the modern dark-field microscope.

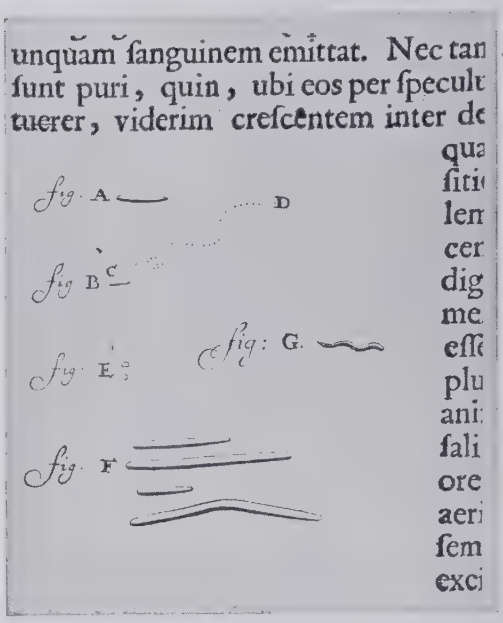


Figure 1. The first pictorial representation of bacteria. (Reproduced from *Arcana Naturae delecta ab Antonio van Leeuwenhoek.* Delphis Batavorum apud Henricum Crooneveld. 1695.)

The direct demonstration of living organisms of such small dimensions was a notable achievement, but any relation of such forms to natural phenomena such as fermentation or infectious disease either escaped van Leeuwenhoek or was of no interest to him. Their possible relation to gross phenomena was apparent to at least some, and possibly many, scientific men of his day. For instance, in a comment on a report of an epidemic disease of cattle published 5 years later, his contemporary, Slare, wrote[8]:

> I wish Mr. Leeuwenhoek had been present at some of the dissections of these infected Animals, I am perswaded he would have discovered some strange Insect or other in them.

But systematic study was delayed for many years, and it was nearly a century

later, in 1786, that the Danish zoologist, O. F. Müller, studied the bacteria and succeeded in discovering many details of their structure. He left drawings so accurate that the bacteria he showed can be identified today as belonging to one or another of the chief divisions. Somewhat later, in 1838, Ehrenberg published his work *Infusionstierchen* in which he put the study of these microorganisms on a systematic basis and was able to establish a number of groups by clearly recognizing fundamental morphological distinctions, such as those differentiating the spirochetes from certain of the protozoa with which others had grouped them. Some of the names he used, such as "bacterium" and "spirillum," are still current in bacteriological nomenclature though with somewhat changed significance. In the next two or three decades considerable knowledge was amassed by such men as Dujardin, Perty, Nägeli, Cohn, and others. It is of more than passing interest that Cohn was Koch's professor.

The perfection of the modern compound microscope, with achromatic and then with apochromatic and fluorite objective lenses, markedly facilitated the study of the morphology of microorganisms, and at least certain of the gross internal structures of the bacterial cell as well as some of the larger viruses could be observed. This made possible accurate differentiation of microorganisms on a morphological basis and provided the basic criteria for characterization of the larger forms, the fungi, protozoa, and metazoa. The introduction of phase microscopy in the 1940's further facilitated certain aspects of microscopy, especially the observation of microorganisms in the living state, by accentuation of slight differences in the refractive indices of intracellular elements.

Optical microscopy is, nevertheless, limited by the resolution that may be obtained with visible light to a working limit in magnification of 1000 to 2000 diameters and resolution of objects as small as 0.2 μ. It was obvious that such resolution limits are inadequate for study of much of the intracellular structure of bacteria and, further, that living organisms beyond the limits of optical resolution existed. The darkfield microscope, although facilitating the observation of tenuous microorganisms such as the spirochetes and structures such as flagella, shows minute objects only as points of light against a dark background without increasing resolution. The use of ultraviolet light in substantially the conventional compound microscope equipped with quartz lenses resulted in some small increase in resolving power in the 1920's, but the fundamental limitation of the size of the object in relation to the wave length of the light used still remained.

Within the next decade or two the electron microscope was developed in practical usable form. It constituted an important advance in microscopy in that an object or structure casting a "shadow" in the electron beam may be resolved, and working magnifications of 30,000 diameters with excellent resolution were obtained. Thus it became possible to photograph microorganisms beyond the limits of optical resolution and to demonstrate directly morphological characteristics down to and including the orientation of the macromolecules that represent the complete unit of certain of these agents.

SPONTANEOUS GENERATION

For many years it was generally believed that living organisms could arise *de novo* and fully formed from decomposing organic matter. The development of snakes from horse hairs standing in stagnant water and the appearance of mice in decomposing fodder are familiar examples that, in fact, persist in some areas to the present time. The fallacies in such beliefs were suspected by some, and in the seventeenth century a number of individuals carried out experiments designed to show whether living organisms had their origin only in other

living organisms (biogenesis), or appeared spontaneously in decomposing organic matter (abiogenesis).

The poet physician Redi carried out experiments in the middle of the seventeenth century that showed, contrary to popular belief, that maggots were not formed spontaneously in decomposing meat but were fly larvae hatched from eggs deposited in the meat. Spallanzani, an Italian monk, showed further that putrescible meat infusions did not spoil when properly heated and did not contain living organisms even though kept over long periods. Needham, an Irish priest, took issue with Spallanzani on the basis of similar experiments in which spoilage took place and living organisms appeared in spite of previous heating. A second series of elaborate experiments by Spallanzani corroborated his earlier findings and indicated the fallacies in Needham's experiments. It was evident that the source of microorganisms was air, and this was convincingly demonstrated by many other workers, including Schulze, Schwann, Schröder and von Dusch, and Tyndall.

The whole question seemed settled conclusively in favor of biogenesis when it was raised again in the middle of the nineteenth century by the work of the eminent French chemist Pouchet. He made the same kind of technical errors as some of his predecessors had, so that his evidence supported the hypothesis of spontaneous generation of life. It was at this point that Pasteur entered the renewed controversy through his early studies on fermentation, and he was able to show beyond any reasonable doubt that the source of microorganisms was air by demonstration of the varying numbers of microorganisms in air from various sources, and by ingenious experiments in which the heated material had free access to air through tortuous passages that allowed bacteria-laden dust to settle before the air reached the putrescible test materials.

The validity of biogenesis has not since been seriously questioned. While biogenesis may seem self-evident today, it was a matter of fundamental importance to biology that it be fully established. Had this not been done the specific microbic etiology of fermentation, decay, infectious disease, and similar phenomena could not have been established. At the same time present concepts of evolution, and biochemical evidence in particular, seem to point inevitably toward an original emergence of life from the nonliving (Chap. 3).[9]

FERMENTATION AND BIOCHEMICAL PHYSIOLOGY

By the middle of the nineteenth century the general nature of organic material was becoming relatively clear, but the natural decomposition of these substances was not, in that the part played by microorganisms in the processes of putrefaction, decay, and fermentation was not known. This is something of an inconsistency in that in some of the earlier studies on spontaneous generation, the decomposition of meat infusions was taken as evidence of the presence of living organisms and, since the organisms were not found in the absence of decomposition, a causal relation was suggested.

The plantlike nature of yeast had been shown by Caignard-Latour and by Schwann, but the chemists of the day, Liebig, Berzelius, and Wöhler, regarded the presence of yeast cells in a fermenting mixture as no more than incidental to the decomposition which was considered to have a purely inanimate basis.

Pasteur (1822–1895), originally trained as a chemist, had done his early work on stereoisomerism. The formation of optically active amyl alcohol during the course of the lactic acid fermentation led him to the study of the processes of fermentation. Having first established the validity of biogenesis, it was not difficult to prove that fermentations resulted from the physiological activity of living, growing microorganisms. It is now clear, of course, that while the living organism is an indispensable adjunct to fermentation, catalysis of the process

is a function of the enzymes formed by the living cell and may be brought about by cell-free preparations.

The specificity of fermentations, the fact that different kinds of fermentations characterized by differences in predominant end products result from the activities of different kinds of microorganisms, grew out of this work and was the beginning of the establishment of the important principle of specific microbic etiology. Pasteur applied this in his work on the "diseases" of beer and wine and showed these were none other than secondary fermentations brought about by extraneous microorganisms that gave undesirable end products. He controlled the fermentative process by gentle heating to destroy undesirable microorganisms, followed by inoculation of the fermentable mixture with microorganisms that brought about the desired fermentation. This method of gentle heating, now also widely applied to destroy pathogenic microorganisms that may be present in milk, has been named "pasteurization" in his honor.

Study of the mechanisms of fermentation was tremendously stimulated by the commercial value of end products such as ethanol, lactic and acetic acids, glycerol, butanol, and acetone on the one hand, and on the other by the obvious similarities in the metabolism of carbohydrate by many microorganisms and by higher animals, including man. The former consideration led to the establishing of a new industry concerned with the large scale production of organic solvents and subsequently other microbial products, especially vitamins and antibiotics.

The chemistry of microorganisms, necessitated by their morphological simplicity and physiological complexity, grew side by side with the chemical approach to mammalian physiology, eventually fusing into present-day biochemistry. As indicated above, the initial common ground was carbohydrate metabolism and the respiratory processes, astonishingly closely similar in such widely dissimilar organisms. Thus, the phenomenon of anaerobic respiration, first observed by Pasteur in fermenting mixtures and received with incredulity at the time, is now commonplace in biochemical physiology. More detailed study showed that the catalysis of the processes of respiration is substantially the same also, and characterization of the iron porphyrin and flavoprotein enzymes, for example, was markedly facilitated by their availability in microorganisms. Many of the vitamins required by mammals, especially those of the B group, are also required by microorganisms, and apparently function in the same manner, *i.e.*, as prosthetic groups or precursors of essential enzyme systems. Similarly, many microorganisms require preformed amino acids in much the same way that the mammal does.

In other respects the physiological potentiality of microorganisms goes far beyond that of any other living organisms. One gets the impression that these forms constituted a testing ground for a variety of physiological machinery, only some kinds of which have had sufficient survival value to persist on any large scale through the evolutionary process. While mammalian metabolism is simulated by some microorganisms, others are photosynthetic and resemble the green plants in the photochemical reduction of carbon dioxide or differ in that the photochemical reactions are coupled with the metabolism of inorganic sulfur compounds. Still others are chemoautotrophic in that they derive energy for the reduction of carbon dioxide from the oxidation of inorganic substrates such as hydrogen, or inorganic compounds of nitrogen, sulfur, iron, and manganese, and the nitrogen-fixing bacteria assimilate atmospheric nitrogen either alone or in symbiosis with leguminous plants.

Their sorting out by Winogradsky, Beijerinck, Hellriegel and Wilfarth, and others, beginning around the turn of the century, represents a remarkable achievement, not only in the isolation of physiological types of microorganisms for which there was no precedent, but also in the elucidation of the mechanisms of the cyclic transformations of the elements in

nature and the microbial basis of soil fertility. Thus the autotrophic nitrifying bacteria, uniquely characterized by the oxidation of ammonia to nitrite, and of nitrite to nitrate, make possible the complete cycle of transformation of nitrogen compounds. The nitrogen leaking from the fixed form is more than replaced by microbial fixation, and the nature of the rejuvenation of depleted soil by cultivation of legume crops is made clear.

INFECTIOUS DISEASE

Sometimes new vistas are opened prematurely and not appreciated at the time, or too late and are anticlimactic, but occasionally new concepts coincide with an unusually receptive segment of the general stream of thought and seem to strike fire. The elucidation of the causes and possible prevention, control, and cure of rampant killing infectious diseases, with which almost every person was intimately familiar, was one of the last, and caught popular imagination as it developed.

The transition from the specific microbic etiology of fermentations and related phenomena to that of infectious disease was more readily reached than might appear. The analogies between the fermentable mixture and the susceptible individual, between active fermentation and clinical disease, and finally subsidence of active fermentation to recovery from and immunity to infectious disease were obvious. Further, the spread of contagion was already appreciated and something of the nature of the infectious agent indicated by epidemiological evidence as in Snow's studies on cholera, and those of Semmelweiss and Holmes on puerperal sepsis in hospitals.

The implications of Pasteur's studies on fermentation for infectious disease were almost immediately appreciated, notably by Lister, the British surgeon. He applied the basic principles to his own work, controlling infection in the operating room by liberal use of phenol, and so initiated, in 1867, an era of antiseptic surgery with a remarkable reduction in intercurrent infection and mortality. These practices were displaced within the next two decades by those of aseptic surgery, largely by von Bergmann in Berlin, as a consequence of a growing appreciation of the importance of infected persons as the primary source of sepsis.

It remained for the German physician Koch (1843–1910) to develop the experimental methods necessary to proof of a causal relation between bacteria and infectious disease. From the first applications of microscopy to the study of microorganisms it was clear that different morphological types existed and that these occurred in nature in mixed populations for the most part. It was evident also from the early studies on pyemia that morphological criteria alone did not suffice to differentiate and characterize the bacteria since morphologically identical forms differed markedly in pathological properties. It was essential to separate such microorganisms from one another.

Initially this had been accomplished approximately, as in Pasteur's earliest work, by dilution in liquid culture mediums, and tubes showing growth in high dilutions of inoculum in which the occurrence of growth was irregular in replicates were assumed to contain only one kind of bacterium, descendants of the single viable parent cell present in the inoculum. Probably one of the greatest single contributions to technical bacteriology was the method of isolation in pure culture developed by Koch. It consisted of dilution of the inoculum and culture on a nutrient medium solidified as a gel by the addition of gelatin, and later agar, that served to separate viable cells from one another with the consequence that their progeny developed as discrete masses of cells of single ancestry, a *clone* in zoological terminology. Modern microbiology was in large part made possible by, and is based upon, this simple technique.

Although the fungus causing favus was described by Schoenlein in 1839,

and the yeastlike organism of thrush by Langenbeck in the same year, the specific microbic etiology of infectious disease was first clearly established in studies on anthrax, an epidemic and highly fatal disease of cattle and other domestic animals. The microorganism now known as *Bacillus anthracis* had been observed by Davaine and Rayer in 1850 and by Pollender in 1849 in the blood and organs of animals dying of anthrax, and the disease was transmitted by Brauell in 1867 by the inoculation of normal animals with infected blood. It is generally conceded, however, that modern medical bacteriology began with Koch's studies on this disease in 1877, for in them he developed conclusive evidence of the causal relation of the anthrax bacillus to the disease of a kind subsequently known as satisfying Koch's postulates, *i.e.*, isolation of the microorganism in pure culture, reproduction of the disease in experimental animals by inoculation with the pure culture, and demonstration of the microorganism in the experimental disease. Thus the kind of evidence required to establish the etiology of infectious disease was delineated, and, when shortly thereafter the solid medium technique for the ready isolation of bacteria in pure culture was developed, the last technical block fell into place.

These advances, coupled with the development of staining methods by Koch, Ehrlich, Weigert, and others, provided a tremendous stimulus to the study of infectious diseases that resulted in an immense accumulation of new knowledge within the ensuing two decades. Koch isolated the tubercle bacillus in 1882 and the cholera vibrio in 1883; Klebs described the diphtheria bacillus in 1883, and it was isolated by Löffler in the following year; Fränkel discovered the pneumococcus in 1886, and the meningococcus was isolated in 1887 by Weichselbaum. Kitasato cultivated the tetanus bacillus in 1889, and in 1894 he and Yersin discovered the plague bacillus independently. The discovery of diphtheria toxin in 1888 by Roux and Yersin and of tetanus toxin by Kitasato in 1889 made possible some insight into the means by which bacteria produce disease.

Characterization of the pathogenic bacteria in morphological, physiological, and pathological terms, and study of their persistence under adverse conditions and behavior in the infected host were obvious and essential corollaries. Out of this kind of information there grew an understanding of the basic elements of the spread of infection from man to man, and from animal reservoirs of infection to man, either directly as in tuberculosis or indirectly as in bubonic plague, to give a firm foundation to epidemiology. Thus, while Snow deduced the presence of the causative agent of Asiatic cholera in the feces of diseased persons and its transmission to others by way of a common contaminated water supply in the famous Broad Street Pump epidemic in London in 1854, the nature of water-borne enteric disease became much more clear with the isolation and study of enteric pathogens such as the cholera vibrio and the typhoid and dysentery bacilli.

Effective control was an inevitable consequence of the developing understanding of infectious disease and its dissemination. The application of indicated control measures, chlorination of water supplies, pasteurization of milk, etc., coupled with widespread utilization of methods of artificial immunization, has been astonishingly successful. It has resulted in the virtual disappearance of many of the great killing diseases, such as smallpox, typhoid fever, Asiatic cholera, diphtheria, and plague, and a tremendous reduction in others, such as tuberculosis and scarlet fever, in many parts of the world. All of this stems in large part from implementation of the principle of specific microbic etiology of infectious disease.

VIRUSES

Early in the study of the etiology of infectious diseases it became apparent

that there were etiological agents of disease, apparently living, in that the diseases could be transmitted indefinitely in series, which could neither be seen with the light microscope nor cultivated apart from living host cells, *i.e.*, were not cultivable on the lifeless mediums that supported the growth of bacteria. Iwanowski in 1892, and Beijerinck in 1899, observed the first of these agents, that causing mosaic disease of the tobacco plant, which Beijerinck described as a *contagium vivum fluidum* since it was present in bacteria-free filtrates of infected juice. A similar agent causing foot-and-mouth disease of cattle was described in 1897 by Löffler and Frosch, and the causative agent of yellow fever by the American Army Commission under the direction of Reed in 1900. Similar agents producing a transmissible lysis of bacteria were found by Twort in 1916 and d'Herelle in 1917, to give three groups of agents now known as the plant, animal, and bacterial viruses respectively.

The term virus steadily contracted from its meaning of any living agent, including bacteria, that was current prior to 1930, through the limitation filterable (or filtrable) virus because some of these agents would pass bacteria-proof filters, to the present significance of the term after about 1940. The extremely small size of the viruses was inferred from both inability to see them and from the ability of at least some to pass through fine filters.

Over the next four or five decades accumulated information, based first on the ability of viruses to pass through some but not all collodion membranes of graded porosity, sedimentation rates in high speed centrifuges, and finally direct observation with electron microscope, showed that these agents ranged from a diameter to 200 mμ, or just at the limit of resolution of the light microscope, to infectious particles as small as 10 mμ. Further, chemical analyses showed that the larger forms contained protein, polysaccharide, and lipid in biologically reasonable proportions and thus resembled bacteria, while the smaller forms appeared to be pure nucleoprotein. Some of the latter were, in fact, prepared in crystalline form; the first was tobacco mosaic virus which was prepared as needle-shaped paracrystals in 1935. Subsequently other plant viruses were prepared in the form of true crystals, *viz.*, tomato bushy stunt virus as uniform rhombic dodecahedral crystals, and in 1955 a strain of the poliomyelitis, one of the smallest animal viruses, was prepared as bipyramidal tetragonal prisms. From data such as these, it was obvious that the viruses do not constitute a homogeneous group of infectious agents.

Related microorganisms, perhaps to be regarded as intermediary between the bacteria and the viruses, were described as the causative agents of a number of febrile diseases. The etiological agent of spotted fever was found by Ricketts in 1909, and subsequently da Rocha Lima, von Prowazek, and others showed that similar microorganisms caused fevers of the typhus group, tsutsugamushi and related diseases, and still later Q fever was found to be of similar etiology. These microorganisms, known as rickettsiae, were found to be as large as small bacteria and so demonstrable under the light microscope but, like the viruses, to proliferate only in the presence of appropriate host cells.

As a consequence of this complete dependence of the viruses and rickettsiae on living host cells, a variety of methods for their propagation in tissue culture were developed since the early part of the twentieth century. In such tissue cultures the host cells were found to be affected adversely, the cytopathogenic property of viruses, and it has been possible to demonstrate in this way the presence of viruses in materials from apparently normal human beings, especially from the upper respiratory tract and the intestinal tract. Propagation of viruses in the embryonated hen's egg was described in the early 1930's as proliferation on the chorioallantoic membrane, in the allantoic cavity or yolk sac, or in the tissues of the embryo itself depending upon the agent and the route

of inoculation. With the advent of antibiotics in the 1940's, it became possible to control the contamination of cultures of animal cells and tissues, resulting in the development of readily applicable methods of tissue culture for the propagation of viruses. The stimulus to virology was similar to that given to bacteriology by Koch's method of isolating bacteria in pure culture.

This dependence upon the host cell characterizing the viruses and rickettsiae raised the concept of parasitism to a high degree of intimacy. With one or two minor exceptions, these agents have been found to have no independent metabolism and thus to parasitize the metabolic mechanisms of the host cell, and some of them, particularly the bacterial viruses, appear to consist of little more than genic material which, on entry into the host cell, dominates its normal directive mechanisms to force the synthesis of new virus substance. It then became possible to show that nucleic acids, separated from the virus particle by phenol extraction or other methods, gave rise to the synthesis of complete virus on introduction into the host cell. Such preparations are known as infectious nucleic acid. The fundamental significance of such observations is obvious, and it has become more and more difficult, if indeed it is possible, to differentiate between the living and the nonliving.

IMMUNITY

It had long been a matter of common knowledge that the individual recovered from infectious disease was often specifically refractory to subsequent attacks of that disease, and that this refractory state, or acquired immunity, persisted in some instances for many years. The practice of deliberate exposure to the infectious agent in order to produce the disease, and therefore immunity, persists to this day in the case of a few diseases such as mumps and measles which are milder and less complicated in children than in adults. It reached a culmination of a sort in the practice of variolation, the deliberate inoculation of a susceptible person with pustular material from an individual with smallpox (variola). The disease so produced was less often fatal than naturally acquired smallpox and provided a more or less permanent protection against it. Variolation had been practiced in the Near East for an unknown time prior to its introduction to Western Europe in 1718 by Lady Mary Wortley Montague, wife of the British ambassador in Constantinople.

Artificial inoculation with an infectious agent of reduced virulence for man to produce a mild infection and consequent protection against naturally occurring disease began with the work of the Englishman Jenner. Noting the infrequent occurrence of smallpox in milkmaids who had been infected with cowpox (vaccinia), he undertook deliberate inoculation of man with vaccinia, followed by inoculation with variola some weeks later. He was able to report in 1796 that such prior infection conferred a high degree of protection against smallpox. This method of vaccination, or Jennerian prophylaxis, was widely practiced thereafter and remains in use today.

With the discovery of pathogenic microorganisms, it became possible to study immunity to infectious disease in a systematic way. Pasteur, working first with chicken cholera and later with anthrax and swine erysipelas, showed that the basic principle uncovered by Jenner could be generalized to include disease other than smallpox. His most striking application of it was the development of rabies prophylactic, rabies virus that had been reduced in virulence for man by successive passages in the rabbit. Not long afterward the American workers, Salmon and Theobald Smith, found that inoculation with killed microorganisms could also stimulate the development of immunity. In 1890 von Behring and Kitasato laid the basis for antitoxic immunity through their discovery of tetanus and diphtheria antitoxins. This rounded out the approach to effective prophylaxis of infectious disease by ac-

quired immunity produced in response to the artificial inoculation of modified microorganisms, killed microorganisms, or relevant products of microorganisms.

It became evident that the immune animal is characterized by, first, the presence of antibody, reacting specifically with microorganisms and their products, in the body fluids and localized in the globulin fraction of the serum protein, and second, by an accelerated response of the phagocytic cells to the presence of microorganisms. The former, humoral immunity, was studied intensively by many workers such as Bordet, Ehrlich, and others, and two general approaches may be distinguished. The one has been concerned with the relation of humoral antibody to effective immunity. In some cases, such as antitoxic immunity, this seemed clear, but in others such as antibacterial and antiviral immunity, the immune state is more complex and presents many problems that are still unsolved.

The other has been concerned with the nature of antigenic specificity, which was elucidated largely through the work of Landsteiner in the 1920's, the nature of antibody, and that of the antigen-antibody reaction in physiochemical terms. Serology, the *in vitro* study of antigens and antibodies, led to the development of diagnostic serological reactions such as the Widal and Wassermann tests, and made possible the characterization of microorganisms on the basis of their constituent antigens by application of the methods of antigenic analysis.

The study of immunity associated with the activity of phagocytic cells, or cellular immunity, was developed initially largely by Metchnikoff in the late 1880's and thereafter. Proceeding from studies with *Daphnia*, he expanded the phenomenon of engulfment and destruction of invading microorganisms by polymorphonuclear leucocytes (microphages) and macrophages into a system of immunology. On this foundation Aschoff described and defined the free and fixed tissue phagocytes as the reticulo-endothelial system of cells. Further elucidation of the developmental potencies of cells of lymphoid origin by Maximov led to a general conception of the inflammatory reaction and cellular response to infection as a process of mobilization of phagocytic cells and destruction of microorganisms, followed by tissue repair through the development of macrophages into fibroblasts.

While cellular immunity and humoral immunity developed somewhat apart, it was early apparent that they are both facets of the host defense mechanism, obviously linked by the occurrence of circulating opsonic antibody, and by the intimate relation of cells of the lymphoid-macrophage system to antibody formation.

CHEMOTHERAPY

One of Paul Ehrlich's many contributions to microbiology around the turn of the century was that to the chemotherapy of infectious disease. He urged the idea of the "magic bullet," a chemical compound, nontoxic to the host, that would search out and kill the invading microorganisms. Substances having a selective antimicrobial activity were known, but had arisen from folklore, or later on a trial-and-error basis not sanctified by age. Quinine used for the treatment of malaria and representing the active principle of cinchona bark is a notable example of the former, and exploitation of the small antimalarial activity of methylene blue leading to the synthesis of Atabrine is an instance of the latter. Ehrlich implemented this concept by the synthesis and testing of hundreds of compounds of arsenic and eventually arrived at the parent compound salvarsan, which was an effective chemotherapeutic drug for the treatment of African sleeping sickness and syphilis. The antimicrobial activity of antimony was similarly followed out by others to give compounds that are reasonably effective in the treatment of infections with some of the animal parasites, but for many years all efforts to prepare antimicrobial

substances effective in the treatment of bacterial infections were fruitless.

In retrospect it is clear that the fallacy was the unquestioned acceptance of the working hypothesis requiring that a substance, to have promise, must have bactericidal activity. It became apparent later, and only since the early 1930's, that a bacteriostatic effect inhibiting reproduction of the invading microorganism is sufficient to tip the balance in favor of the host, with actual destruction of the bacteria accomplished by the host defense mechanisms.

Probably the first rational approach to the biological aspects of the problem of the chemotherapy of disease of bacterial etiology was that of Avery in the late 1920's. He put two kinds of information together. The one was the ability of microorganisms in general to decompose any kind of organic substrate, and the other the intimate association between the presence of capsular polysaccharide substance in the pneumococcus and the ability of that bacterium to cause disease. It seemed possible to isolate a microorganism that would specifically decompose the pneumococcal polysaccharide and separate the enzyme, catalyzing that decomposition for use as a therapeutic agent. This was accomplished but, while the cell-free enzyme preparation had therapeutic activity, it was too highly toxic to the host to have practical value.

A variation on a similar theme grew out of the study of microbial antagonisms, the adverse effect of one kind of microorganism on another when grown together in mixed culture. This effect was found to be due, in some instances, to the formation of a specifically toxic substance by the one kind of microorganism. The first of these found, pyocyanin, had in fact been known since the middle of the nineteenth century, prior to the work of Pasteur, Koch, and others, and had been extracted with organic solvents from the "blue pus" characterizing infections with the microorganism forming it.

The great majority of such substances, including pyocyanin, were found to be highly toxic to higher animals, but some proved to be of sufficiently greater toxicity to the microorganism than to the host that effective concentrations could be produced and maintained in the tissues of the infected host. Of these substances, the antibiotics, the first to be discovered was penicillin which was found in 1929, but its possible chemotherapeutic activity was not appreciated until 1940; prior to that time its greatest utility was that of facilitating the primary isolation of microorganisms, such as the influenza bacillus, that are relatively resistant to it. Of the other more widely known antibiotics, streptomycin was found in 1943, chloramphenicol in 1947, and the tetracylines in 1948, 1950, and 1954. Although resting on a firm biological background, the discovery of these substances still remains on trial-and-error basis in that they are found through methods of screening vast numbers of microorganisms for activity.

The discovery of the chemotherapeutic activity of sulfonamides in the 1930's was also accidental. More than 1000 derivatives of the parent compound, sulfanilamide, have been prepared, of which perhaps half a dozen are in common use. Since these substances antedated the discovery of the antibiotic chemotherapeutic agents, they were the first effective antibacterial drugs, and the effect of the introduction of these substances was nowhere more dramatic than in the treatment of puerperal sepsis. The fundamental importance of the sulfonamides, however, did not become apparent until 1940.

During the preceding decade a body of detailed information relative to the nutritional requirements of bacteria had been assembled, and study of the nature of the antibacterial activity of the sulfonamides had shown that it was primarily bacteriostatic rather than bactericidal. These apparently unrelated kinds of information were joined in 1940 by Woods's observation that the antibacterial activity of sulfonamides was specifically antagonized by *p*-aminobenzoic acid, an essential growth factor for some

bacteria and utilized by very many if not all in the synthesis of folic acid. The structural similarity of *p*-aminobenzoic acid to the active portion of the drug molecule, *p*-aminobenzene sulfonic acid, suggested that the antibacterial activity of the latter was a result of a specific interference with the synthesis of an essential metabolite. This proved to be true and provided the first rational theory of chemotherapy of infectious disease in establishing the general principle of using structural and functional analogues of essential metabolites to exploit small differences, qualitative or quantitative, between the essential metabolic reactions of the host and parasite cells. Its general significance is illustrated in the chemotherapy of neoplastic disease with nucleic acid analogues.

MICROBIAL GENETICS

Variability of microorganisms was obvious from the beginning of their systematic study as alterations in certain of their properties, produced inadvertently as a consequence of maintenance on artificial culture mediums or as changes deliberately induced by laboratory manipulation. Alteration in the properties used for the differentiation and characterization of microorganisms was found to occur with some facility, not as a consequence of an unusual plasticity, but rather because of their short generation time and occurrence as populations made up of huge numbers of individuals. Because of the relative simplicity of structure of microorganisms, especially the bacteria and viruses, the bacteriologist lacked a comparative anatomy that makes up the foundation of botany and zoology. He was forced to rely on such seemingly ephemeral characteristics as fermentation, nutritive requirements, and antigenic character, all of which varied, and had no means of knowing whether any of these, with the possible exception of the last, were biologically trivial or fundamental.

Studies in the 1920's with yeasts showed that, in these forms at least, the ability to ferment various carbohydrate substrates was genically determined in that a Mendelian segregation of characters of this kind occurred in the exchange of nuclear material during spore formation. This work was largely neglected by bacteriologists, and the demonstration that conjugation occurred between physiologically unlike bacterial cells did not occur until the 1940's.

While the importance of establishing the occurrence of genic control of the kind of characters used for the differentiation and identification of bacteria is unquestionable, microbiology contributed uniquely to, and markedly broadened, the basis of heredity through the occurrence of phenomena hitherto not demonstrable in other forms.

In 1928 it was shown that immunological types of living pneumococci could be changed by growing them in the presence of killed pneumococci of another type. Further refinement made it clear that the active principle in the dead pneumococci was a highly polymerized deoxyribonucleic acid that could be separated from the cells and purified in the test tube before addition to the living culture. This phenomenon was later found to occur also with other kinds of bacteria and is possibly related to the mechanisms of replication of certain of the bacterial viruses whose "invasion" of the host cell was found to consist of "inoculation" of that cell with viral deoxyribonucleic acid, in that in both kinds of phenomenon foreign nuclear material becomes functional in directing a distortion of the metabolism of the host cell so that it synthesizes a new substance and, in the case of immunological transformation, breeds true.

Still another kind of modification of hereditary mechanisms was described in the early 1950's in which bacterial virus was found to transmit from one bacterial cell to another physiological characters that appear as stable inheritable elements in the new host cell. Finally, it has long been known that alteration in the physiological behavior of bacteria may be induced specifically by the envi-

ronment, even in the absence of cell multiplication, and this phenomenon of enzyme adaptation is conditioned by the specific substrate of each enzyme.

Contributions such as these have made it possible to break away from the concept of direction of cell metabolism as invariably linked with the classic gene-chromosome structure. It appears, rather, that the molecular pattern of the determinant may be introduced into the recipient physiological system in a variety of ways, and may even be purified in the test tube, and yet retain its directional function.

RECONSIDERATION

The microbiologist has been something of a renegade, by necessity a master of many trades but a servant of none, and showing a certain lack of respect for the classical and established. Pasteur was not impressed with the opinions of Liebig and Berzelius, nor was it of any concern to Koch that no self-respecting medical man would work with a disease of domestic animals. The microbiologist has taken what he needed from zoology, botany, chemistry, physiology, pathology, medicine, etc., to create a new discipline, and it is apparent from even a cursory survey of the more important developments tracing its growth over a century that remarkable results were achieved.

It is literally true that the majority of the important infectious diseases were conquered in that they have been, or can be, so effectively controlled as to remove all but tuberculosis from the ten leading causes of death in this and certain other countries, and thus has been made probably the largest single contribution to increased human life expectancy. New industries have been created, notably the fermentation industry with its ramifications into the production of antibiotics and vitamins of microbial origin, and the biologicals and chemotherapeutic drugs of the pharmaceutical industry. Less obvious, but more important, has been a large group of unique contributions to biology, ranging from the creation of the new science of immunology, through much of biochemical physiology, to a greatly broadened concept of hereditary mechanisms.

Aside from such highly successful practical and theoretical applications, microbiology owes its important position in biological science to its general significance. There is no question but that microbiology has produced a change in man's conceptions of the world around him so sweeping as almost to deserve the term revolutionary. Up to the middle of the nineteenth century the character of many of the most familiar natural processes was entirely misunderstood; contemporary spontaneous generation of at least the lower forms of life was the generally accepted belief; infectious diseases were not differentiated from one another, and the most fantastic hypotheses were advanced to explain their existence.

Although the great mass of material phenomena elsewhere had been brought into apparent orderliness and system, here was a region in which the unscientific imagination rioted in mystery and extravagance. The penetration of this realm of obscurity by the discoveries of microbiologists gave the human race for the first time in its history a rational theory of disease, dispelled the myths of spontaneous generation, and set the process of decay and kindred phenomena in their true relation to the great cycle of living and nonliving matter.

The new conception of the microscopic underworld which microbiology brought into biological science must be reckoned as a conspicuous landmark and, insofar as it has changed the attitude of man toward the universe, may be regarded as one of the most important triumphs of natural science.

REFERENCES

1. Brock, T. D. (ed.): Milestones in Microbiology. Prentice-Hall, Englewood Cliffs, New Jersey. 1961.

2. Bryson, V. (ed.): Microbiology: Yesterday and Today. Institute of Microbiology, Rutgers University, New Brunswick, New Jersey. 1959.
3. Bulloch, W.: The History of Bacteriology. Heath Clark Lectures, 1936. Reprinted Oxford University Press, New York. 1960.
4. Clark, P. F.: Pioneer Microbiologists of America. University of Wisconsin Press. 1960.
5. Dobell, C.: Antony van Leeuwenhoek and His "Little Animals." Harcourt, Brace & Company, New York. 1932.
6. Grainger, T. H.: A Guide to the History of Bacteriology. Chronica Botanica, Int. Biol. Agr. Ser. No. 18. The Ronald Press, New York. 1958.
7. van Leeuwenhoek, A.: Observations, communicated to the publisher by Mr. Antony van Leeuwenhoek, in a Dutch letter of the 9th of Octob. 1676, here Englished: Concerning little animals observed by him in rain- well- sea- and snow water, as also in water wherein pepper had lain infused. Phil. Trans. Roy. Soc., 1677, **11–12:**821.
8. Slare, F.: An abstract of a letter from Dr. Wincler, chief physitian of the Prince Palatine, Dat. Dec. 22, 1682 to Dr. Fred Slare, Fellow of the Royal Society, containing an account of a murren in Switzerland, and the method of its cure. A further confirmation of the above mentioned contagion, of its nature, and manner of spreading by way of Postscript from the ingenious Fred Slare, M. D. and F. R. S., Dat. March 27, 1683. Phil. Trans. Roy. Soc., 1682, **13:**93.
9. Symposium: Modern ideas on spontaneous generation. Ann. N. Y. Acad. Sci., 1957, **69:** 255–376.

Chapter 2

LABORATORY METHODS*

THE STUDY of microorganisms in the laboratory by the various procedures of isolation in pure culture, cultivation, microscopic examination, and characterization by biochemical and immunological methods necessarily begins before detailed consideration of their nature and properties can be completed. The basic laboratory procedures are summarized here in didactic form, and their rationale will become apparent later.

STERILIZATION

The determination of the character and properties of a given species of microorganism is necessarily based on its study in pure culture, *i.e.*, its separation from other microorganisms. Since bacteria and other microorganisms such as fungi are ubiquitous, all material coming in direct contact with the microorganisms under study must be subject to preliminary sterilization. The sterilizing agent most commonly used is heat, dry or moist, but occasionally other methods, such as filtration of liquids through filters which retain microorganisms cultivable in the usual laboratory mediums, are desirable.

* For the most part the methods described here are identical with or closely follow those of generally accepted procedures to be found in various compilations included among the references at the end of this chapter.

Glassware and Instruments. The usual glassware includes flasks, petri dishes, test tubes and pipettes which must, of course, be scrupulously clean. Flasks and tubes are plugged with nonabsorbent cotton which prevents entry of bacteria after sterilization, and a cotton plug is inserted in the mouth end of the pipettes.

Petri dishes and pipettes may be placed in cans with covers or wrapped in paper to maintain sterility. Surgical instruments are wrapped in paper or towels, syringes separated and wrapped in paper, hypodermic needles placed in plugged test tubes, and other equipment similarly prepared.

Sterilization is effected in a hot air oven, electric or gas fired. The material to be sterilized is placed in the oven without crowding and the temperature raised to 170° to 180° C. and maintained for a period of not less than two hours. If appropriate temperature control equipment is not available, a slight browning of the cotton plugs is taken as indicative of sterilization, but this is a doubtful procedure.

In some instances sterilization by heating to dull red in a Bunsen flame is exceedingly useful as in the flaming of forceps tips, the platinum or nichrome wire needles and loops used for transferring bacteria, and the lips of test tubes before and after transfer of bacterial culture.

Surgical instruments, hypodermic needles and syringes, and similar equipment may be sterilized with respect to the vegetative forms of bacteria by boiling in water or 1 per cent bicarbonate solution for 3 to 5 minutes, but this does not suffice to destroy the spores of bacteria and fungi.

Culture Mediums and Other Liquids. Material containing water cannot, of course, be sterilized by dry heat, but moist heat is a more effective sterilizing agent in any case.

Intermittent sterilization. As indicated before, boiling water does not provide a high enough temperature to bring about complete sterilization. Certain kinds of liquid culture mediums, however, are affected unfavorably by moist heat at higher temperatures, and these may be sterilized by intermittent sterilization.

This is based on the assumption that the vegetative cells of microorganisms are destroyed at 100° C. in the presence of water, and that the surviving spores will germinate, and these vegetative cells will be destroyed in turn by exposure to 100° C. In practice the material to be so sterilized is exposed to free-flowing steam in an Arnold sterilizer for 30 minutes, removed and incubated until the next day and the steaming repeated, and a third incubation and steaming is included as a safety factor so to speak. The method is not too successful in that spores not infrequently show a delayed germination, and spores of obligate anaerobic bacteria may not germinate. Sterilization by the intermittent method is, however, seldom required.

Autoclave sterilization. The most efficient method of sterilization is that effected by steam at temperatures above 100° C. which may be produced when the steam is under pressure. The devices employed in the use of steam under pressure include the common pressure cooker, autoclaves of various designs, and dressing sterilizers, which are autoclaves to which vacuum can be applied in order to dry fabrics that have been sterilized. Steam is generated directly in the apparatus or supplied by connection to a high pressure steam line.

As in the case of dry heat sterilization, the material to be sterilized must not be packed too tightly in the autoclave. The temperature is raised to 120° C. and maintained for a period depending on the material to be sterilized. As short a time as 15 minutes suffices for small volumes of material, as in culture tubes, loosely spaced, but if the tubes are packed in baskets, 20 minutes or possibly more should be allowed. For larger volumes, as in flasks, longer periods of exposure are necessary, *e.g.*, for 500 ml. quantities 30 to 40 minutes. The time is that elapsing after the temperature is reached and until the pressure is shut off; the preliminary heating as pressure is built up and the slow fall in pressure at the end are not included. No attempt should ever be made to reduce the pressure rapidly after sterilization, for liquids will boil out of their containers.

Sterilization by filtration. It is often desirable to sterilize solutions such as cultures in liquid mediums, solutions of substances relatively unstable to heat such as certain sugars and the like, without subjecting them to heat at sterilizing temperatures. These may be sterilized by filtration through filters of such fine porosity that most microorganisms are held back. The method of sterilization by filtration is particularly useful in obtaining soluble products such as toxins.

Several kinds of filters are available. The Berkefeld filters are made of infusorial earth in three porosities, V (*viel*) or coarse, N (*normal*), and W (*wenig*); of these the V filters remove most but not all bacteria, the N filters usually sterilize, and the W filters are used for very small microorganisms. The Chamberland filter, of unglazed porcelain, is made in graded porosities, L1, L2, L3, etc., and of these the L3 is roughly equivalent to the Berkefeld N. The Seitz or Seitz-Werke filter is made of metal, and the filtering element is an asbestos pad which is the equivalent of the Berkefeld N filter. Sintered glass filters are available in a variety of porosities which in the Pyrex filters are designated C (coarse), M (medium), F (fine), and UF (ultrafine). The UF filter is bacteria-proof while the others are not. Cellulose ester membrane filters, marketed as Millipore filters, are available in a variety of pore sizes; type HA, with a pore diameter of 0.45μ, is sterilizing filter.

Filtration is accomplished by a pressure differential which may be obtained by positive pressure on the liquid to be filtered, or negative pressure on the filtrate, and usually a differential of 20 to 30 cm. Hg suffices for reasonably rapid filtration without foaming. The Berkefeld and Chamberland types are prone to develop leaks, either as cracks in the filter candle, or where the candle is cemented to the metal portions, and the integrity of the filter must be controlled frequently by including a trace organism such as *Serratia marcescens* in the liquid to be filtered and by culture of the filtrate. Whatever filter is used it must, of course, have been sterilized, usually in the autoclave, prior to use.

PREPARATION OF CULTURE MEDIUMS*

The nutritive requirements of microorganisms vary greatly; some will grow readily in the so-called synthetic mediums containing inorganic salts, including an ammonium salt, together with a simple organic compound such as glucose or asparagin as a source of carbon and energy. In general, microbiological mediums have been developed on a trial-and-error basis about a basic nutrient medium containing peptone and the water-soluble material, largely extractives, of muscle tissue. The source of the latter may be commercial preparations of meat extract, but a somewhat better medium is obtained if these substances are extracted from fresh meat, beef or veal for the most part.

The basal nutrient solution may be modified in a variety of ways. Thus, it

* Many culture mediums are available commercially in dehydrated form, requiring only solution in water, dispensing, and sterilizing. Certain commonly used agar mediums are similarly available as poured plates or slants in plastic disposable "glassware."

may be solidified by the addition of gelatin or agar, or enriched with serum, ascitic fluid, defibrinated blood, and the like, to support the growth of the more fastidious bacteria. Various sugars may be added, together with an acid-base indicator, for the determination of the fermentative properties of bacteria; nitrate or tryptophan may be added to test for ability to reduce nitrate to nitrite, form indol from tryptophan, etc. Or culture mediums may be prepared which are differential and accentuate physiological differences, and specific inhibitory agents may be added to produce selective mediums which allow the growth of some kinds of microorganisms and suppress that of others. These various characteristics may be combined, of course, as in the case of an enriched medium which is both selective and inhibitory. Consequently, there is a great variety of bacteriological culture mediums, but they are in large part interrelated modifications of a basal nutrient solution. The constituents and method of preparation of a few of the commonly used culture mediums are reviewed here.

Basal Mediums

Meat extract broth and agar. The medium usually has the following composition:

meat extract	3 gm.
peptone	5 gm.
(NaCl	5 gm.)
(agar	15 gm.)
distilled water	1000 ml.

It is necessary to include the sodium chloride if the medium is to be used for serological work or enriched with blood, and the agar is omitted if a liquid medium is desired. The ingredients are dissolved in water. Agar has the property of not dissolving or of its gels not melting unless it is heated to boiling, but its solution does not gel until the temperature reaches about 40° C. If agar is used it is necessary to boil the solution or heat it in the autoclave and clarify by filtration. The initial solution is usually slightly acid, and the reaction is adjusted to pH 7 by the addition of alkali.

Meat infusion broth and agar. The infusion mediums differ from the extract mediums in that the extractives are obtained by infusion of fresh meat, but otherwise the composition is the same, *viz.*,

extract of 400 to 600 gm. fresh lean beef or veal	
peptone	5 gm.
(NaCl	5 gm.)
(agar	15 gm.)
distilled water to	1000 ml.

The meat is ground in a meat chopper, suspended in 1 liter of distilled water, and infused overnight in the refrigerator. The following morning the fat is skimmed off with absorbent cotton and the infusion squeezed through muslin and made up to 1000 ml. The other ingredients are added, the reaction adjusted to pH 7, the solution heated to 100° C. for about 20 minutes, regardless of whether or not agar is used, filtered through coarse paper, and made up to 1000 ml. The heating is for the purpose of coagulating the tissue proteins dissolved during infusion so that they can be removed by filtration.

Mediums for Biochemical Tests

Sugar broths. The sugar broths are meat extract or meat infusion broth to which the required carbohydrate has been added to a concentration of 1 per cent. Unless the carbohydrate is stable to autoclave sterilization, it must be sterilized separately by filtration in concentrated solution and added aseptically to the sterile broth. It is customary to add an acid-base indicator dye, bromthymol blue or bromcresol purple, and the medium may be dispensed in culture tubes containing an inverted vial that fills during sterilization and serves to collect gas evolved in the fermentation of the sugar.

Nitrate broth. To test for ability to reduce nitrate, bacteria are cultured in nitrate broth, infusion or extract broth containing 0.1 per cent KNO_3. The culture is tested for the presence of nitrite with sulfanilic acid and α-naphthylamine reagents.

The sulfanilic acid reagent is prepared by dissolving 8 gm. of sulfanilic acid in 1000 ml. of 5N acetic acid. The α-naphthylamine reagent (α-amidonaphthalene acetate) is prepared by dissolving 5 gm. of α-naphthylamine in 1000 ml. of 5N acetic acid and clearing by filtration through absorbent cotton.

To test for nitrite add 2 ml. of each of the two reagents to 3 to 5 ml. of culture; the development of a rose color, the nitroso reaction, indicates the presence of nitrite.

Tryptophan broth. The test for the production of indol from tryptophan can often be carried out with cultures in meat broth or peptone water, the latter containing peptone but no meat extract or infusion. The important factor is an adequate amount of tryptophan in the peptone. Some peptones are deficient, but a peptone marketed under the name Tryptone contains adequate amounts of the amino acid.

The presence of indol is tested for after 2 to 4 days' incubation of the culture by layering a small amount of Ehrlich's reagent on the surface of the culture; the development of a red color at the interface indicates the presence of indol. If a

color does not appear within 1 minute, add a small amount, equal to that of the Ehrlich reagent, of saturated aqueous solution of potassium persulfate.

Ehrlich's reagent consists of 4 gm. of *p*-dimethylaminobenzaldehyde dissolved in a mixture of 380 ml. of ethyl alcohol and 80 ml. of concentrated hydrochloric acid.

Lead acetate agar. This medium is meat extract or meat infusion agar containing 0.05 per cent basic lead acetate and dispensed in culture tubes without slanting. It is usually prepared by mixing equal amounts of sterile double strength basal medium, *i.e.*, containing double amounts of the ingredients, and sterile 0.1 per cent aqueous solution of basic lead acetate.

The medium is inoculated by stab, and the production of hydrogen sulfide from the sulfur-containing amino acids of the peptone is indicated as a blackening or browning of the medium.

Meat extract and meat infusion gelatin. These mediums are the basal mediums solidified by the addition of 12 per cent gelatin and are dispensed in culture tubes without slanting and inoculated by stab. They indicate whether or not gelatin is liquefied by the microorganism under study. Gelatin liquefies at 37° C., and gelatin cultures should be incubated at 22° C. In the case of those bacteria which will not grow at this lower temperature, the culture may be incubated in the usual way and after incubation tested for ability to solidify by chilling. Gelatin liquefaction sometimes occurs slowly, and cultures should be retained for not less than 2 weeks unless positive earlier.

Milk. Milk is an adequate culture medium for many bacteria without modification other than the addition of an indicator, bromcresol purple or litmus in an amount of 5 ml. of a 0.25 per cent alcoholic solution per liter. Fresh skim milk may be used, but skim milk powder is usually more convenient. One liter of medium is made from 150 gm. of milk powder, first rubbed up in a mortar and then diluted to volume. Medium prepared from powdered milk may be autoclaved, but that from fresh milk is best sterilized by the intermittent method.

Because of protein and carbohydrate content milk cultures undergo a variety of biochemical changes, *viz.:*

(1) The development of an alkaline reaction, usually after 3 to 4 days' incubation.

(2) The development of an alkaline reaction with precipitation of the casein as a rennet curd, with or without reduction of the indicator.

(3) The development of alkalinity and rennet curd precipitation, followed by peptonization or digestion of the curd, resulting in a clearing and brownish discoloration; the indicator is usually reduced by the time digestion is apparent.

(4) Acid formation, usually in 24 hours, with or without reduction of the indicator.

(5) Acid formation and precipitation of the casein, usually with reduction of the indicator.

Enriched Mediums. Basal infusion mediums may be made richer by the use of special peptones, such as neopeptone and proteose peptone, and the addition of small amounts of glucose and phosphate buffer. In addition, fluids such as serum, ascitic fluid, or defibrinated blood may be added.

Blood agar. The most common and most useful enriched medium is blood agar—veal or beef infusion base to which defibrinated whole blood is added. The blood is usually taken from the rabbit, sheep or horse under sterile conditions and defibrinated as it is drawn by shaking with glass beads. The sterile infusion agar base is liquefied by heating, cooled to about 45° C., the sterile blood added to 10 per cent concentration, and the medium dispensed before it solidifies, usually in petri dishes or as slants in culture tubes. The entire operation is carried out under sterile conditions and the medium incubated for 24 hours to detect contamination, and then stored in the refrigerator. This medium is rich enough to support the growth of almost all the fastidious pathogenic bacteria and has the advantage that hemolysis is shown directly in the cultures.

Cystine blood agar. This is simply blood agar medium further enriched by the addition of 0.01 per cent cystine and 1 per cent glucose to the basal medium prior to the addition of blood as above. It is useful primarily in the cultivation of *Pasteurella tularensis.*

Chocolate agar. This medium is heated blood agar and is particularly useful for the cultivation of the gonococcus and meningococcus. Formulas vary somewhat, some workers preferring a beef heart infusion (see below) instead of muscle tissue infusion and proteose peptone instead of peptone. The basal infusion medium is enriched by the addition of defibrinated blood as in the preparation of blood agar, but after the addition of the blood it is heated slowly in a water bath until the medium has a chocolate brown color; if the heating is excessive the blood coagulates and the finished medium will contain clumps of cooked blood, so the temperature should not rise above 75° C. at the most.

Other infusions. The use of infusions of tissues other than beef or veal muscle is sometimes desirable for the cultivation of certain bacteria. Beef liver infusion is prepared in the same manner as that described for the other infusions and, for culture of Brucella for which liver infusion medium is particularly useful, the basal medium is enriched by the addition of 1 per cent egg albumin. Beef heart infusion is also prepared in the same manner as beef muscle infusion, and the basal medium completed by the addition of a peptone, sodium chloride, etc.

Löffler's medium. This medium is used primarily for culture of the diphtheria bacillus and consists of 3 parts of sterile beef serum and 1 part of meat infusion basal medium containing

1 per cent glucose. The serum is added to the sterile base, mixed, dispensed in culture tubes, slanted, and sterilized by the intermittent method. Sterilization may be carried out on 3 successive days in the autoclave at 15 pounds steam pressure without letting the air out, or it may be held at 15 pounds for 15 minutes and the air allowed to escape slowly while pressure is maintained and, when all the air has escaped, holding for an additional 15 minutes, and after sterilization allowing the pressure to fall very slowly. The medium is solid because of coagulation of the serum, and sterilization must be carried out carefully to avoid the formation of bubbles.

Blood culture medium (Kracke). This is a highly enriched medium containing beef heart infusion and brain suspension and is used primarily for blood culture of pathogenic bacteria in infections accompanied by bacteremia. Its composition is:

heart infusion	750 ml.
brain suspension	250 ml.
sodium citrate	1 gm.
dextrose	10 gm.
peptone	10 gm.
dibasic sodium phosphate	2 gm.
sodium chloride	4 gm.

The heart infusion is prepared in the same manner as muscle tissue infusion described earlier. The brain suspension is prepared by macerating 500 gm. of fresh brain in water, straining through a metal strainer, and heating slowly to boiling with constant stirring. The heating coagulates the brain tissue and leaves it in a state of fine suspension; it must not be filtered. The other ingredients are added to the mixture of heart infusion and brain suspension, the pH is adjusted to 7.4, the mixture is dispensed in 50 ml. amounts in 100 ml. flasks (to leave room for an inoculum of 10 to 20 ml. of patient's blood) and sterilized by autoclaving. Cultures in this medium are essentially enrichment cultures and should be subcultured on appropriate agar mediums such as blood agar for streptococcoci and staphylococci and on liver infusion agar for Brucella.

Dorset's egg medium. This medium is used for the cultivation of the tubercle bacillus and consists of 4 fresh eggs and 25 ml. of 0.95 per cent sodium chloride solution. The eggs are scrubbed with a brush in soap and water, rinsed, dried, put into a wire basket and dipped into 95 per cent alcohol, drained, and the remaining alcohol ignited. They are then broken, using aseptic technique, and the whites and yolks placed in a sterile container. Twenty-five ml. of sterile salt solution is added, the whole mixed well with a sterile egg beater, dispensed in culture tubes and slanted, and sterilized by the intermittent method described for Löffler's medium.

Differential and Selective Mediums. The various differential and selective mediums have been designed to facilitate the isolation of one kind of bacteria in the presence of many other kinds, by accentuating physiologic differences and/or inhibiting the growth of the unwanted forms. Of the pathogenic bacteria, those most commonly encountered admixed with large numbers of closely related forms are the enteric bacilli, and many of the selective mediums are selective for these bacteria. The simplest of the purely differential mediums consist of meat extract or infusion agar base together with a sugar and an indicator. The most satisfactory indicator in most cases is bromthymol blue, and the colonies of the sugar-fermenting bacteria are yellow while those of the nonfermenters are blue. Of the selective agents bacteriostatic dyes are often used, and others include bile, tellurite, etc.

Endo's medium. This medium consists of meat extract agar to which has been added lactose and Schiff's reagent, basic fuchsin decolorized with sulfite. When the lactose is fermented aldehyde intermediates restore the color to the fuchsin and colonies of lactose-fermenting bacteria are red while those of the non-lactose-fermenters are white. The composition of the medium is:

hot melted extract agar	1000 ml.
sodium carbonate, 10% aqueous	10 ml.
lactose	10 gm.
sodium bisulfite, 10% aqueous	10 ml.
basic fuchsin, 3% alcoholic	10 ml.

The pH of the medium is raised by the carbonate and should be 7.6 to 8.0.

Eosin–methylene blue agar. This medium is sterile meat extract agar to which is added 5 ml. of a 10 per cent solution of lactose, 2 ml. of 2 per cent aqueous eosin, and 2 ml. of 0.5 per cent aqueous methylene blue. These solutions are added aseptically to the melted and cooled medium, and the mixture is dispensed in sterile petri dishes.

Desoxycholate–citrate medium. This medium is differential in that it contains lactose and neutral red as an indicator, and selective in that it contains bile salt, and is one of the most satisfactory and widely used mediums for the isolation of Salmonella and dysentery bacilli. It contains:

meat infusion	1000 ml.
peptone	10 gm.
agar	20 gm.
lactose	10 gm.
sodium citrate	20.6 gm.
sodium desoxycholate	5 gm.
lead chloride, 0.35% aqueous	1 ml.
ferric ammonium citrate	2 gm.
neutral red, 1% aqueous	2 ml.

The complete medium is not stable on storage. The peptone and meat infusion are mixed, the pH adjusted to 7.5. The solution is boiled for 3 minutes, lost water added, and filtered through paper. The agar is added to the hot solution together with 5 ml. of N NaOH, and it is allowed to stand for 15 minutes, then steamed at 100° C.

for 20 minutes. Then the lactose, citrate, desoxycholate, and lead chloride are added. This may be stored and remelted for use. Just before use, the agar is heated to 100° C., the ferric ammonium citrate added, and the pH adjusted to 7.4 using phenol red as an indicator, and finally the neutral red is added and the medium dispensed in plates. Note that it is not autoclaved.

Bismuth sulfite medium (Hajna). This medium is used primarily for the isolation of the typhoid bacillus from fecal specimens and is highly specific for that organism. As originally developed it was difficult to prepare consistently, but Hajna's modification is highly satisfactory. It consists of a meat extract agar base containing 0.5 per cent glucose, the bismuth sulfite mixture, and brilliant green. The bismuth sulfite mixture is prepared as follows:

(1) Dissolve 80 gm. of anhydrous bismuth sulfite in 400 ml. of hot water with stirring.
(2) Make a paste of 24 gm. of bismuth citrate in 40 ml. of water, add 12 ml. of concentrated ammonia, and stir until a sol is formed. Dilute to 200 ml. with distilled water and mix to give a solution.
(3) Mix (1) and (2), and add 42 gm. of anhydrous dibasic sodium phosphate and mix until dissolved.
(4) Dissolve 4 gm. of ferrous sulfate in 50 ml. of distilled water containing 2 drops of concentrated hydrochloric acid, and add 40 ml. of this solution to mixture (3). Mix and boil gently for about 2 minutes until slate gray in color. This is the bismuth sulfite mixture, and it is stable for about 2 months.

For use, add 70 ml. of the bismuth sulfite mixture and 4 ml. of a 1 per cent aqueous solution of brilliant green to each 1000 ml. of the hot melted agar base and autoclave for not more than 10 minutes; then dispense in petri dishes. The complete medium may be kept for perhaps 2 weeks in the refrigerator without autoclaving and should be autoclaved just prior to dispensing.

Tetrathionate enrichment broth. Nutritive enrichment broths are used for preliminary culture of specimens followed by subculture on selective differential mediums. Tetrathionate broth and selenite F broth (see below) are very useful in the isolation of Salmonella from fecal specimens. The active agents in tetrathionate broth are bile salts, brilliant green, and tetrathionate, the last formed by oxidation of thiosulfate with iodine just prior to inoculation. Its composition is as follows:

proteose peptone	5 gm.
bile salts (Bacto)	1 gm.
distilled water	1000 ml.
calcium carbonate	10 gm.
sodium thiosulfate	30 gm.
brilliant green, 1% aqueous	11 ml.
iodine, 25% aqueous	2.5 ml. per 100 ml.
iodine	25 gm.
potassium iodide	20 gm.
water	100 ml.

The peptone and bile salts are dissolved in water and calcium carbonate added, and autoclaved. The sodium thiosulfate and brilliant green are added to the sterilized medium, and it is dispensed in tubes or small flasks. Just prior to inoculation add the iodine solution in the proportion of 2.5 ml. per 100 ml.

Selenite F broth. This medium contains selenite as an inhibitory agent and its composition is:

sodium hydrogen selenite	4 gm.
peptone	5 gm.
lactose	4 gm.
sodium phosphates (anhydrous)	10 gm.
distilled water	1000 ml.

It is necessary to determine the proportions of acid and basic phosphates which will give a final reaction of pH 7.0 to 7.1 with the peptone and the lot or brand of selenite used. The ingredients are dissolved in warm water and then brought to a boil and dispensed. The medium is not autoclaved.

Chocolate-tellurite medium. This is a chocolate agar prepared by adding 10 per cent of defibrinated blood to meat infusion base agar as described earlier but modified by the addition of 150 ml. of sterile 0.3 per cent aqueous solution of potassium tellurite to every 1000 ml. of infusion base. It is used for culture of diphtheria bacilli.

Petragnani's medium. This is a medium used for culture of tubercle bacilli and contains malachite green to inhibit the growth of gram-positive bacteria and molds. Its composition is:

milk	900 ml.
potato flour	36 gm.
peptone	6 gm
pieces of potato, size of an egg	6
eggs	24
egg yolk	6
glycerin	72 ml.
malachite green, 2% aqueous	60 ml.

The potatoes are cut into thin slices, the milk, potato flour, and peptone added, and the whole cooked in a double boiler for 2 hours with stirring. The eggs and egg yolks are broken together, the glycerin and malachite green added, and the mixture shaken well. The milk-potato mixture is cooled to 50° C., the egg-glycerin-dye mixture added and mixed well, and the whole is filtered through gauze, dispensed into tubes, slanted, and sterilized by the intermittent method described for Löffler's medium.

MICROSCOPIC EXAMINATION

The direct observation of microorganisms is an essential part of their study. Characteristics such as the shape and

grouping of the cells, the presence or absence of structures such as capsules, flagella, and spores, the reaction to differential stains, and the like, are of considerable differential significance.

Motility

Some bacteria have flagella which function as organs of locomotion and are analogous to the cilia of protozoa. The absence, or presence and number and location of flagella, is a characteristic of differential value. Flagella are too small to be seen in the usual stained preparations, but may be demonstrated by special staining procedures in which dye is deposited on them. The demonstration of flagella by staining methods gives results which are too uncertain to be useful as a routine procedure. Their presence, though not number and position, may be inferred from a demonstration of motility, *i.e.*, a movement of translocation as differentiated from the random motion of brownian movement. This may be carried out in two ways, *viz.*,

(1) Motility may be observed directly in suspensions of young cultures (usually not more than 18 hours old) suspended in a drop of saline or broth on a coverglass inverted on a hollow ground or "well" slide (the hanging drop preparation). Unstained bacteria are difficult to see, and the amount of light should be reduced to a minimum to give maximum resolution. Observation is facilitated by focusing the microscope on the edge of the drop and then moving the slide so that the drop is within the field of observation.

(2) Motility may also be demonstrated by stab culture in "motility medium," a soft (0.5 per cent) agar nutrient medium dispensed in culture tubes. The medium is inoculated with the straight wire in a single stab penetrating several centimeters into the medium. Following incubation, the growth of nonmotile bacteria is found to be confined sharply to the path of the inoculating needle, while that of motile bacteria tends to diffuse out from the line of inoculation to give a fuzzy streak of growth.

Staining

Bacteria may be stained or dyed with aniline dyes, more readily with the basic dyes.[5] Staining may be with a single dye, or simple stain, most commonly crystal violet, methylene blue, or basic fuchsin, with mixed dyes or polychrome stains, or by differential methods based on the relative affinity of different bacteria or different structures of the bacterial cell for the stains used.

The so-called Romanovsky or polychrome stains are relatively complex mixtures of dyes that stain the constituents of protoplasm in contrasting colors —generally nuclear material red and cytoplasm blue. They are used primarily for staining blood and blood parasites such as trypanosomes and malarial parasites. In general, they consist of oxidation products of methylene blue (methylthionin) together with the parent substance and the compounds formed between these basic dyes and the acid dye eosin (eosinates). Of these, Giemsa stain is one of the most widely used. American Giemsa contains Azure A (asymmetrical dimethylthionin) eosinate, Azure B (trimethylthionin) eosinate, methylene blue eosinate and methylene blue chloride dissolved as a stock solution in methyl alcohol and glycerol. The material to be stained is commonly fixed in methyl alcohol, and stained by flooding with a freshly prepared 1:10 dilution of stock solution of Giemsa.

The nature of the staining process has been a matter of considerable controversy, in large part as to whether it is fundamentally a physical or chemical process. It is now generally agreed that it is chemical in nature and the mechanism an ionic interchange between the basic dye and the acidic portions of the protoplasm—nucleic acids and their compounds—with the formation of insoluble dye-nucleotide compounds that do not diffuse out of the cell.

Stain Solutions. The composition of stain solutions varies considerably in the literature. The several, frequently somewhat indefinite, formulas have been interpreted by the Biological Stain Commission and are given here in the emended form recommended by that Commission. In older formulas the dye content is often given as milliliters of a saturated alcoholic solution, and the accompanying table gives the solubilities of those most commonly used, in water and in 95 per cent alcohol.

Solubilities of Common Bacteriological Stains

COLOR INDEX NUMBER	NAME	PER CENT SOLUBLE IN WATER	PER CENT SOLUBLE IN 95% ALCOHOL
655	Auramine O	0.74	4.49
681	Crystal violet (chloride)	1.68	13.87
676	Fuchsin, basic (pararosanilin hydrochloride)	0.26	5.93
657	Malachite green (oxalate)	7.60	7.52
922	Methylene blue (hydrochloride)	3.55	1.48
841	Safranin	5.45	3.41
925	Toluidine blue O	3.82	0.57

Löffler's alkaline methylene blue

Solution A

methylene blue (90% dye content)	0.3	gm.
ethyl alcohol (95%)	30	ml.

Solution B

dilute KOH (0.01% by weight)	100	ml.

The two solutions are mixed in the above quantities.

Ziehl's carbolfuchsin

Solution A

basic fuchsin (90% dye content)	0.3	gm.
ethyl alcohol (95%)	10	ml.

Solution B

phenol	5	gm.
distilled water	95	ml.

Mix the two solutions in the above quantities.

The Gram stain

Ammonium Oxalate Crystal Violet (Hucker)

Solution A

crystal violet (90% dye content)	2	gm.
ethyl alcohol (95%)	20	ml.

Solution B

ammonium oxalate	0.8	gm.
distilled water	80	ml.

Mix the two solutions in the above quantities.

Safranin

safranin (2.5% solution in 95% alcohol)	10	ml.
distilled water	100	ml.

Albert's diphtheria stain (Laybourn)

toluidine blue	0.15	gm.
malachite green	0.02	gm.
acetic acid (glacial)	1	ml.
ethyl alcohol (95%)	2	ml.
distilled water	100	ml.

Preparation of Smears. Slides should be clean and free from all grease, and may be cleaned in soap and water, thoroughly rinsed, and stored in alcohol or xylol. Before use a slide is passed through the Bunsen burner flame two or three times. A drop of distilled water is placed on the cooled slide and a small amount of bacterial growth suspended in it, spread, and allowed to dry in air. The film is then fixed by passing through the burner flame two or three times, or, for some stains, by immersion in absolute methyl alcohol, glacial acetic acid, or other fixatives.

Procedure of the Simple Stain. The fixed smear is covered with stain, allowed to stand for 30 to 60 seconds, rinsed off with tap water, excess water removed by blotting, and allowed to dry in air. Bacteria are usually examined under the oil immersion objective, and the oil can be placed directly on top of the smear.

The Gram Stain. Of the differential stains, the Gram stain is one of the most valuable and most generally applied. The procedure is essentially one of staining with crystal violet, mordanting with iodine solution, decolorizing with alcohol, and counterstaining with a dye of contrasting color. The stain was originally developed by the histologist Gram in an effort to stain bacteria in tissues differentially and it has been subject to many modifications. By this procedure bacteria are separated into two groups, those which retain the crystal violet and are said to be *gram-positive*, and those which are decolorized and stain with the counterstain and are *gram-negative*. The distinction is not always sharp for there is considerable variation in ease of decolorization, some gram-positive bacteria such as the pneumococcus become gram-negative after they die,

and some bacteria are gram-variable. This last group is not large enough to detract from the practical value of the stain.

Both the crystal violet and the iodine are highly specific while the alcohol and the counterstain are not. Almost all other dyes, even methyl green which differs by only one methyl group from crystal violet, are unsuitable, either being retained or removed by alcohol whether or not the iodine is applied. A few other reagents, notably $HgCl_2$, may be substituted for iodine, and stannous chloride, pyrogallol, and freshly prepared solutions of hydroquinone show some slight activity.

However arbitrary Gram's method of staining may appear, the reaction is apparently associated with fundamental differences between the gram-positive and gram-negative organisms. There is, for example, a pronounced correlation between it and resistance to the bacteriostatic action of certain dyes and antibiotic substances and to the action of other chemical and physical agents. The nature of the Gram reaction has, therefore, been of considerable interest (see Chap. 3).

Procedure of the Gram stain. In addition to crystal violet and safranin staining solutions, Gram's* iodine solution is required as a mordant. Its composition is:

iodine	1 gm.
potassium iodide	2 gm.
distilled water	300 ml.

The staining procedure is as follows:

(1) The heat-fixed smear is stained for 1 minute with ammonium oxalate crystal violet.
(2) Wash in tap water.
(3) Flood with Gram's iodine solution and allow to stand 1 minute.
(4) Wash in tap water and blot dry.
(5) Decolorize 30 seconds with gentle agitation in 95 per cent alcohol, and blot dry.
(6) Counterstain 10 to 30 seconds in safranin.
(7) Wash in tap water, blot dry, and examine.

Acid-fast Stain (Ziehl-Neelsen Method). Certain bacteria, characterized by a high lipid content, cannot be stained by the usual procedure of the simple stain, and either heat or prolonged contact is required to drive the stain into the cells. Conversely, the stained forms are equally difficult to decolorize and resist decolorization with acid alcohol. These organisms are designated acid-fast and include the tubercle bacilli and related forms, the leprosy bacillus and certain of the actinomycetes.

Staining procedure

(1) Stain the smear for 3 to 5 minutes in steaming Ziehl's carbolfuchsin. An alternative procedure useful in diagnostic laboratories is to stain in the cold for 18 to 24 hours.
(2) Rinse in tap water.
(3) Decolorize in 95 per cent ethyl alcohol, containing 3 per cent by volume of concentrated hydrochloric acid, until only a suggestion of pink color remains.
(4) Wash in tap water.
(5) Counterstain for 30 to 60 seconds with alkaline methylene blue.
(6) Wash in tap water, dry, and examine.

Fontana Stain for Spirochetes. The spirochetes stain very poorly or not at all by the usual simple staining procedure and are usually stained by a silver impregnation method.

Preparation of ammoniacal silver nitrate solution. Dissolve 5 gm. silver nitrate in 100 ml. of distilled water. Remove a few milliliters, and to the rest of the solution add, drop by drop, concentrated ammonia solution until the sepia precipitate which forms is dissolved. Then add, drop by drop, enough more of the silver nitrate solution to produce a slight cloud which persists after shaking. This solution is stable for some months.

Staining procedure

(1) Steam the heat-fixed smear in a solution of 5 per cent tannic acid in 1 per cent phenol for 30 seconds.
(2) Wash for 30 seconds in running water.
(3) Flood with ammoniacal silver nitrate solution, heat gently, and allow to stand 20 to 30 seconds after steaming has begun.
(4) Wash in tap water, blot dry, and examine. The spirochetes appear as dark brown or black on a dark maroon field.

Capsule Stain (Hiss's Method). The bacterial capsule does not stain in the simple stain or Gram stain procedures. A number of methods of staining the capsules have been used of which that of Hiss is one of the most simple and effective. The stain is an aqueous solution of basic fuchsin or crystal violet, 0.15 to 0.3 per cent and 0.05 to 0.1 per cent respectively.

Staining procedure. The bacteria should be grown in ascitic fluid or serum medium for maximum capsule development, and bacteria grown on solid mediums should be suspended in serum for preparation of the smear. The smear is air-dried and fixed by heat.

(1) Stain with either aqueous basic fuchsin

* This reagent is frequently given as Lugol's iodine; Lugol's iodine is defined in the U.S.P., and in the Gram stain is used as a 1:15 dilution.

or aqueous crystal violet by heating gently until the stain steams.

(2) Wash off the stain with 20 per cent aqueous copper sulfate solution.

(3) Blot dry and examine.

The bacterial cells are deeply stained and the capsules a faint blue or pink, depending upon which stain has been used.

Spore Stain. Like acid-fast bacteria, spores are very difficult to stain and appear as unstained in the usual stained smear. The stain may be driven in by heat, and the Ziehl-Neelsen acid-fast staining procedure may be used with the modification that decolorization is less rigorous, *i.e.*, 95 per cent alcohol or absolute acetone should be used instead of acid alcohol.

CULTURE OF BACTERIA

Methods of Obtaining Pure Cultures. When fluid culture mediums are inoculated with substances such as soil, water, or excreta, many kinds of organisms develop simultaneously side by side, and a heterogeneous mixture, or mixed culture, of bacteria results. Any technical procedure for obtaining pure cultures is dependent upon the isolation of a single viable bacterium which is allowed to multiply in a suitable culture medium. The first reliable method of isolation of pure cultures was devised by Koch in 1881. This method has proved so satisfactory that it has been employed to the present day with only minor modifications. If nutrient gelatin or agar is inoculated while fluid (for example, at 42° C.) and is then solidified and kept under favorable temperature conditions, many of the living bacteria that have been introduced are able to multiply. Since the bacteria cannot move about freely, but are fixed in the stiffened medium, the progeny of each organism form distinct masses or colonies. These colonies consist of many millions of bacteria and are readily visible to the naked eye or by means of a low power hand lens. If the colonies are not closely crowded, a pure culture may be obtained by touching a colony with the tip of a sterile needle and inoculating tubes of fresh culture mediums. In order to secure a large surface upon which the colonies shall be spread out and made easily accessible, the gelatin or agar after inoculation is poured while still fluid into sterilized flat shallow dishes (petri dishes) fitted with glass covers.

It will be clear that a given colony may arise from two or more parent cells if these remain attached or close together in the agar medium, and the colony will not be a pure culture in the event that the juxtaposed cells are of different species. This possibility has been investigated by McNew[6] using the plant pathogen *Phytomonas stewarti*. He found that 98 per cent or more of the colonies arose from single cells, hence the probability of a mixed culture in a single colony is 0.02 or less. Successive plating or picking a number of colonies makes a pure culture a practical certainty. It is likely, however, that this figure differs for different bacteria, *i.e.*, staphylococci are more likely to remain attached than micrococci or bacilli.

It is self-evident that the pure culture so obtained is not only homogeneous with respect to kind or species of bacteria, but since the microorganisms are descendants of a single parent cell, they constitute what the zoologist or protozoologist terms a *clone*.

Technique of making plate cultures. Three tubes of agar (1, 2, 3), melted at 100° C., are placed in a water bath at 42° C., a temperature that is just above the solidifying point of agar and is not injurious to bacteria. Tube 1 is inoculated with a loopful of the material to be plated. The cotton plug is then replaced and the contents of the tube are mixed by carefully tilting back and forth and rotating the tube on its long axis. From this tube two loopfuls of agar are transferred to tube 2, and after mixing, two more loopfuls carried from tube 2 to tube 3. The contents of the several tubes are then poured into petri dishes. As soon as the cotton plug is removed, the mouth of each tube should be passed through the flame and inserted under the edge of the lifted petri dish cover and the agar quickly poured out. The covered petri dish may then be tipped cautiously back and forth to dis-

tribute the agar evenly before it solidifies. Agar plates placed in the incubator after solidification should be inverted in order to avoid spreading of the growth through condensation of moisture on the surface of the medium. Even if there are a great many bacteria in the original material, the plate from tube 3 will probably contain the organisms in small enough numbers to develop well-isolated colonies. On the other hand, if there are very few bacteria in the material inoculated, plate 1 will probably present more satisfactory conditions. Gelatin plates are made in the same manner as agar except that gelatin may be cooled as low as 25° C. without solidifying.

Under exceptional conditions, such as work in the field, when petri dishes are not available, so-called roll tubes may be made. The tubes containing the liquid agar medium are tilted until the agar almost reaches the cotton plug. The tubes are then rotated in this position against a block of ice, and when the process is complete the test tube is coated on the inside with a thin layer of solid medium. In this way a considerable surface is obtained, and, after incubation, colonies are readily picked.

Quantitative dilution. It is sometimes of advantage before plating to make accurate dilutions of highly polluted fluids, such as sewage, in order to get colonies few enough in number to be well isolated. If there is reason to suppose that the number of bacteria is 200 per ml. or more, 1 ml. of the sample is mixed with 9 ml. of sterile water. If a higher dilution is required, proceed in a similar manner. See below.

One milliliter of each dilution is placed in a sterile petri dish, and 6 to 8 ml. of liquid cooled agar medium is added. The two are thoroughly mixed by tilting the petri dish back and forth and then allowed to solidify. After incubation the total number of colonies on the plates containing 50 to 200 colonies is counted, and the total number is multiplied by the dilution to give the number of organisms present in the original sample. Such bacterial counts are relatively inaccurate, errors of 10 to 15 per cent being common in even the most careful work, and must be regarded only as useful approximations.

Streak plates. Not infrequently mediums used for the isolation of the more fastidious bacteria are such that it is technically difficult to make the usual pour plates. The most common of these mediums are those which contain fresh blood. Such mediums are ordinarily prepared in quantity and sterile plates poured. These may be stored in the icebox until used. Dilution and consequent isolation of bacteria may be accomplished with these mediums by a process known as streaking. A sterile wire loop is dipped into the bacterial suspension and rubbed back and forth on the surface of the solid medium, making successive streaks as close together as possible. After incubation it will be found that at some point sufficient numbers of bacteria were rubbed off the loop to allow the development of individual colonies in the path of the streak. These may be picked and transferred to fresh mediums. Quantitative dilutions may be prepared in sterile broth or saline and 0.5 ml. of each dilution pipetted onto the surface of the medium and spread evenly by appropriate tilting.

Carbon Dioxide Tension. A number of bacteria, particularly the gonococcus, meningococcus, and Brucella, require an increased tension of carbon dioxide over that of ordinary atmosphere, commonly 10 to 15 per cent. Cultures of such bacteria are incubated in airtight jars in which a part of the air has been replaced by carbon dioxide. One or another of three methods is commonly used.

(1) The most obvious and effective method is direct replacement in which the jar is a vacuum desiccator that is partially evacuated, 9 to 10 cm. Hg negative pressure, and the evacuated air replaced with carbon dioxide from a tank of the compressed gas. The method has the disadvantage that it requires a certain amount of equipment.

(2) A simple and equally effective method is that of putting a short candle stub in the jar with the cultures, lighting it, and closing the jar. The candle burns for a short time, forming carbon dioxide, and then goes out. The partial pressure of carbon dioxide obtained is not subject to control, but the precise tension is not too important, and adequate amounts are formed.

(3) Carbon dioxide may also be generated from sodium bicarbonate by the addition of sulfuric acid. The amounts required must be calculated on the basis of the size of the jar used so that 10 to 15 per cent of the total gas volume is the evolved carbon dioxide.

Cultivation of Obligate Anaerobes. Atmospheric oxygen is toxic to a group of bacteria designated the obligate anaerobes, which includes the bacilli of

(A)	To dilute 1:		10	use 1 ml. of sample to		9 ml. of sterile water.				
(B)	" 1:	100	"	"	"	" 99	"	"	"	
(C)	" 1:	1,000	"	"	(A)	" 99	"	"	"	
(D)	" 1:	10,000	"	"	(B)	" 99	"	"	"	
(E)	" 1:	100,000	"	"	(C)	" 99	"	"	"	
(F)	" 1:	1,000,000	"	"	(D)	" 99	"	"	"	

tetanus and gaseous gangrene, the botulinus bacillus, the Bacteroides, and anaerobic cocci. Various methods have been devised for the cultivation of these bacteria which center about the elimination of gaseous oxygen and which may be grouped under four general heads.

(*1*) *Enriched Mediums in Deep Tubes.* Perhaps the simplest method is that of the so-called shake culture which is in an agar medium liquefied and boiled to drive out dissolved oxygen, inoculated when cool but still liquid, and solidified quickly in cold water. This is the basis of culture in the Veillon tube, a glass tube stoppered at both ends, which is sometimes used in the isolation of obligate anaerobes in pure culture; the tube may be broken or the column of agar medium forced out into a sterile petri dish and cut at the various points where isolated colonies occur deep in the medium.

A somewhat simpler method of culture in liquid medium is based on the use of a rich medium to which is added a piece of fresh sterile tissue such as rabbit kidney. Such mediums show strong oxygen uptakes as a result of the respiration of the fresh tissue, and the depths of the medium are anaerobic. As soon as growth is initiated the evolution of gas, usually from contained glucose, is vigorous enough to prevent solution of atmospheric oxygen, and the culture remains anaerobic.

(*2*) *Removal of Oxygen by Combustion.* Oxgen may be removed from the gaseous environment of cultures incubated in sealed jars in a number of ways. Sufficiently anaerobic conditions can frequently be obtained by saturating a piece of filter paper with alcohol, placing in the jar and igniting, and sealing the jar immediately. Similarly, a small metal container filled with chalk may be put into a jar, a piece of phosphorus placed in it, and the jar sealed immediately. The phosphorus pentoxide formed will dissolve in a small amount of water placed in the bottom of the jar and is not harmful to bacterial cultures, but the phosphorus begins to burn again when the jar is opened, and the method is somewhat dangerous.

By far the most convenient combustion method is that in which the oxygen is removed by combination with hydrogen in the presence of heated platinized asbestos. Jars designed for this purpose with platinized asbestos heated by a small electric coil have been described by Brewer and are termed Brewer anaerobic jars. The jar rim is sealed against both positive and negative pressure and hydrogen or illuminating gas run in to give some positive pressure. The electric current is turned on, and the oxygen is burned out in the presence of the combustible gas and catalyst. This method is considerably more reliable than the combustion methods described above but is somewhat dangerous; if the platinized asbestos is finely divided, heating is unnecessary.

(*3*) *Chemical Absorption of Oxygen.* Contained oxygen in sealed jars may also be removed by chemical methods, of which the most widely used is a mixture of pyrogallic acid and alkali in the bottom of a vacuum desiccator. The jar should be closed immediately on addition of the reagents, and it is preferable to mix after closing by tilting or spilling one reagent into the other. Excess alkali is to be avoided. The reagents are 20 per cent aqueous sodium hydroxide and 40 per cent aqueous pyrogallic acid mixed in proportions of 5 ml. of alkali to 2 ml. of pyrogallic acid. The alkali–pyrogallic acid method is applicable to single culture tubes; a stopper of absorbent cotton is pushed down to leave about an inch of space above it, pyrogallic acid and sodium hydroxide placed in this space to saturate the cotton, the tube closed at once with a tight-fitting rubber stopper and turned upside down and incubated in that position. It may be used also for the anaerobic incubation of a single petri dish in combination with the Spray dish, which is a false bottom, so to speak, over which the medium-containing half of the petri dish is inverted and sealed. The Spray dish is divided by a ridge in the center; 4 ml. pyrogallic acid is put on one side and 10 ml. of alkali on the other; after sealing on the petri dish bottom, the two are mixed by tilting.

(*4*) *Displacement of Air by an Inert Gas.* The air contained in an anaerobic jar may be mechanically displaced by an inert gas, commonly hydrogen since tanked nitrogen contains sufficient oxygen to inhibit the growth of the fastidious obligate anaerobes. A jar with two connections, one reaching to the bottom, designated a Novy jar, may be used with tanked hydrogen. The hydrogen is run in from the bottom of the jar until the contained air is displaced. The air in an anaerobic jar may also be displaced by hydrogen evolved in the reaction between sulfuric acid and powdered chromium metal.

Control of anaerobiosis. It is usually desirable to include an indicator of anaerobiosis in an anaerobic jar to indicate that anaerobic conditions have been obtained and are maintained, *i.e.*, there is no leakage of atmospheric oxygen into the jar during incubation. The usual indicator is a tube of methylene blue prepared as follows: equal parts of N/160 sodium hydroxide, 0.015 per cent methylene blue and 6 per cent sterile aqueous solution of glucose are mixed, boiled just before the jar is to be sealed until the methylene blue is reduced, and the open tube put into the jar. So long as it remains colorless anaerobic conditions are maintained.

SYSTEMATIC STUDY OF BACTERIA IN PURE CULTURE

The characteristics of bacteria that are of significance in their identification must

be studied in some uniform fashion, not only with regard to the methods used but also as to the characteristics that shall be considered of first importance. Although the classification of bacteria is in a somewhat dubious state, this uncertainty has resulted largely from a relatively hazy comprehension of the fundamental biologic significance of some differential characteristics and consequent doubt as to the proper relative weight to be assigned to them in the arrangement of the finer subdivisions of taxonomic schemes. The main lines of approach, however, are clear, and whatever their relative biologic significance may prove to be, the readily determinable characteristics of bacteria serve the practical purpose of distinguishing these microorganisms.

There are four general categories which, in the order of the fineness of distinction they make possible, are: (*a*) morphology, both gross and microscopic; (*b*) physiologic capabilities in terms of biochemical reactions; (*c*) pathogenicity for experimental animals; (*d*) immunological character.

The preliminary and basic study of a bacterium lies in the systematic determination of its cultural characters. The most important of these are:

(A) Morphology
  (1) gross morphology—that of colonies of the organism with respect to size, texture, color, shape, etc.
    (*a*) on nutrient or infusion semisolid mediums
    (*b*) on special mediums
  (2) microscopic morphology, including
    (*a*) size, shape, and grouping of the organisms
    (*b*) presence or absence of spores
    (*c*) motility
    (*d*) presence or absence of capsule
    (*e*) staining reactions
(B) Biochemical reactions
  (1) the fermentation of sugars, usually dextrose, lactose, and sucrose, although others may be included together with the hydrolysis of starch
  (2) liquefaction of gelatin
  (3) formation of indol
  (4) reduction of nitrate to nitrite
  (5) production of hydrogen sulfide
  (6) special biochemical tests, such as the Voges-Proskauer reaction, the methyl red test, hemolysis on blood agar, etc.

Such preliminary examination ordinarily affords a great deal of information about a given bacterial culture. The feasibility of additional study rests firmly on the foundation laid down by the studies outlined above.

Various attempts have been made to systematize the study of bacteria. One of the most successful of these has resulted from the efforts of a Committee on Bacteriological Technique of the Society of American Bacteriologists (now the American Society for Microbiology).

ANIMAL INOCULATION

An indispensable adjunct to the study of the pathogenic bacteria is the experimental animal. Animals are used not only for the study of the pathology of infectious disease and as an aid in the isolation of some bacteria in pure culture, but also for the experimental production of immune serums and studies on the various manifestations of immune phenomena. The maintenance by animal passage of such infectious agents as the viruses, which cannot be cultivated on lifeless mediums, is common also. The animals generally used are rabbits, guinea pigs, white mice, and white rats, although in special cases others, such as rhesus monkeys, are necessary.

Routes of Inoculation. Experimental animals may be inoculated by a variety of routes, usually one or another being preferable under the particular circumstances. The common routes of inoculation are intradermal, subcutaneous, intramuscular, intraperitoneal, and intravenous. Other routes such as intraocular, intracerebral, and intrathecal may be desirable at times. In any case, inoculation is carried out with a syringe and hypodermic needle, the capacity of the syringe and size of the needle used depending upon the quantity of material

to be injected and the route of inoculation. The site of inoculation must be prepared by removal of the hair by shaving or a depilatory, followed by disinfection by swabbing with alcohol, tincture of iodine, etc. The syringe and needles have, of course, been previously sterilized by dry heat or by boiling.

Intracutaneous inoculation. Material inoculated intracutaneously is inoculated into the skin. A fold of skin is pinched up and the needle inserted, lumen up, as superficially as possible. A raised white spot showing the pits of the hair follicles indicates a successful injection. Not more than 0.2 ml. may be inoculated by this route.

Subcutaneous inoculation. Subcutaneous inoculation requires considerably less skill on the part of the operator. A fold of skin is pinched up as before and the needle inserted through the skin to its full length. The injected material forms a bleb or blister. The amount that may be injected by this route depends upon the size of the animal.

Intramuscular inoculation. In the intramuscular inoculation, the needle is inserted deep into the muscular tissue, usually the posterior muscles of the thigh or the lateral thoracic or abdominal muscles.

Intraperitoneal inoculation. Intraperitoneal inoculation is carried out as one would a subcutaneous injection on the abdomen except that after the needle penetrates the skin, it is held at right angles to the peritoneal wall and thrust through into the peritoneal cavity.

Intravenous inoculation. Intravenous inoculation may be carried out in a variety of ways, the choice depending upon the size of the animal. Rabbits are conveniently inoculated in the marginal vein of the ear. The needle is inserted through the skin and into the vein from the side. If the needle is within the vein, blood will disappear in the vein from the point of the needle onward as the material is injected. Needless to say, the injection should take place in the direction of blood flow. Guinea pigs may be inoculated into the large superficial vein on the dorsal and inner side of the hind leg or into one of the external jugular veins. Both require anesthesia of the animal and a small incision, which may be later closed with a stitch or collodion. Rats may be inoculated in an external jugular vein and mice in one of the lateral veins of the tail.

Drawing Blood. Blood may be taken from the rabbit from the marginal vein of the ear or directly from the heart. The appropriate area is shaved and swabbed with alcohol. If blood is to be taken from the ear, the marginal vein is cut across with a razor blade and the blood allowed to drop into a centrifuge tube or test tube. Flow may be hastened by preliminary application of a towel wrung out of hot water to induce hyperemia. When blood is to be taken directly from the heart, a 30 to 50 ml. syringe is used and the needle inserted between the ribs at the point of maximum pulsation and thrust into the heart, care being taken to injure the pericardium as little as possible. Blood is most conveniently taken from smaller animals by this means.

Postmortem Examination. The autopsy of experimental animals dead of infection is often highly desirable. Not only may cultures be taken, but the gross and microscopic pathology is usually of significance. The animal is fastened down on a board, ventral surface up. It is good practice to have the board in a larger enameled tray to reduce to a minimum the danger of spreading infectious material. When cultures are to be taken, the animal must be opened with sterile instruments. Usually several pairs of scissors and forceps and two or three scalpels are necessary. The site of inoculation is examined for ulcerative or other changes, and the hair and skin are disinfected with cresol or Lysol.

The skin is incised from the pubis to the neck and cut at right angles at the ends of the incision. The two flaps may be laid back by separation of the cutaneous from the underlying muscular tissue with a scalpel. The condition of the subcutaneous tissue and of axillary and inguinal lymph glands may be noted. The peritoneal fluid may be cultured by searing a small area on the surface of the abdomen with a hot spatula and puncturing with a syringe and needle or a Pasteur pipette into which the fluid may be aspirated. The abdominal cavity is opened with fresh instruments, and the flaps are laid back as above. The condition of the abdominal organs, such as spleen and liver, may be noted and cultures taken by first searing the surface and then either puncturing and aspirating fluid with a syringe and needle or a Pasteur pipette, or by removing a small piece of tissue and placing it in nutrient medium. Likewise, pieces of tissue may be removed and placed in fixing fluid for subsequent sectioning. Again with fresh instruments, the costal cartilages are cut to make a V-shaped incision, and this flap is laid back over the head, exposing the thoracic cavity. Any gross pathology may be noted, cultures may be taken from heart's blood and elsewhere if desirable, and pieces of tissue removed for fixation and sectioning. The animal is disposed of by incineration.

It is obviously impossible to define postmortem procedure rigorously. The lymphatic system of guinea pigs dead of tuberculous infection will be of considerable interest. Those dying of diphtheritic toxemia or infection will show characteristic lesions of the adrenals. In some diseases the important postmortem findings may be in the brain and spinal cord. The routine of examination will be determined, therefore, by the particular disease under consideration or, if this is not known, by the symptoms exhibited by the animal before death.

A number of precautions must be observed in the autopsy of animals dead of infectious disease. If cultures are to be taken, fresh sterile instruments must be used at each stage. Instruments once used should be returned to the boil-

ing water sterilizer or placed in a tray containing Lysol or, if to be used again, laid down on the edge of the tray with all sharp edges and points inward. Extreme care must be taken to avoid disseminating infectious material. With certain highly contagious diseases such as tularemia, rubber gloves may be desirable. Boiling water sterilization (five minutes) of used instruments is sufficient except with the spore-forming organisms such as anthrax and tetanus, in which case the instruments should be allowed to stand in a solution of Lysol or cresol.

PROPAGATION OF VIRUSES AND RICKETTSIAE*

The viruses and rickettsiae are set apart from the bacteria by their inability to proliferate except in the presence of living host cells (Chap. 4). The propagation of these microorganisms under experimental conditions in the laboratory, apart from infection of the intact susceptible animal, is dependent upon the use of such living host cells. Appropriate host cells are most readily available as the variety of cells and tissues of the embryonated hen's egg, and as mammalian cells of varied origin maintained as cell or tissue cultures.

THE EMBRYONATED EGG[3, 12]

The sterile, rapidly multiplying, and diversified cells of the embryonated egg include those of the investing membranes as well as those of the embryo proper. The former may be infected by inoculation into the surrounding fluid, as by inoculation of the yolk sac or the allantoic cavity, or directly as in the case of the chorioallantois. The variables, dependent upon the agent being studied, include the age of the embryo, the type of diluent used for the inoculum, the route of inoculation, and the temperature of incubation after inoculation. For example, the typhus rickettsiae are grown in the yolk sac of the 6 to 8 day embryo, influenza virus in the allantoic cavity of the 10 to 11 day embryo, and vaccinia and herpes simplex viruses on the chorioallantoic membrane of the 12 to 13 day embryo. Or the embryo may be inoculated directly, as for instance intracerebrally or intravenously, or may be removed, minced and grown as a tissue culture (see below).

Fertile eggs from disease-free flocks should have white shells to facilitate observation by transillumination. When they are to be used for the propagation of rickettsiae or viruses of the psittacosis–lymphogranuloma venereum group, the flock should not be fed diets supplemented with the broad-spectrum antibiotics. The eggs can be stored, prior to incubation, for as much as a week at 5 to 20° C. with little loss of viability. The eggs are incubated at 37 to 37.8° C. at a relative humidity of 40 to 60 per cent in a forced draft incubator, *i.e.*, a conventional egg incubator. On the fifth day, they may be candled and nonviable embryos and infertile eggs discarded. After appropriate further incubation, the egg may be inoculated by any of several routes.

Yolk Sac. ***Inoculation.*** A small hole is drilled or punched, without rupturing the shell membrane, in the center of the shell over the air space at the large end of the egg. After disinfection of the area with iodine-alcohol, the inoculum is introduced by means of a syringe with a 22 g. 1½-inch needle, which is inserted until the tip of the needle is about in the center of the egg. Amounts of 0.25 to 1.0 ml. can be injected. After the needle is withdrawn, the area around the hole is again disinfected and the hole sealed off with melted paraffin, collodion, or nail lacquer.

Harvest. After disinfection by swabbing with iodine-alcohol, the shell over the air sac is removed with sterile scissors or forceps. The exposed shell membrane and chorioallantoic membrane are cut away, and the contents of the egg tipped into a sterile petri dish, with a long forceps guiding the material. The yolk sac is then dissected away from the other membranes and placed in a second petri dish which has been weighed. After determination of the wet weight of the yolk sac, it is transferred to a sterile flask

* This section was prepared by Dr. Dorothy Hamre.

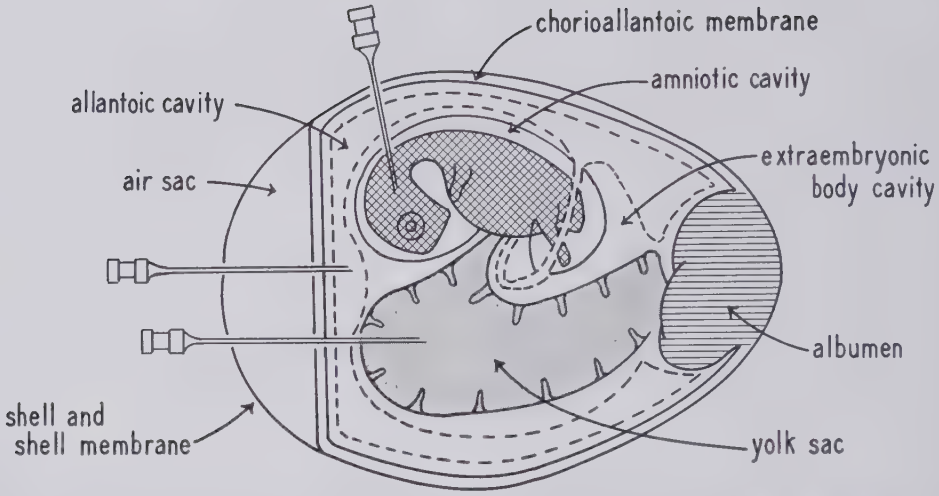


Figure 2. Diagrammatic representation in sagittal section of the embryonated hen's egg 10 to 12 days old. The hypodermic needles show the routes of inoculation of the yolk sac, allantoic cavity, and embryo (head). The chorioallantoic membrane is inoculated after it has been dropped by removing the air from the air sac.

or bottle partially filled with glass beads. A suitable volume of diluent is added, and the flask is closed with a sterile rubber stopper and shaken by hand or on a bottle shaker for about 20 minutes. The resulting suspension is centrifuged to remove cellular debris, and the supernatant fluid, containing the virus, is decanted.

Amnionic Cavity. ***Inoculation.*** The eggs are candled, the air space outlined, and the position of the embryo noted. The shell over the air sac is disinfected and a hole about 1.5 cm. in diameter cut in the center with sterile scissors. The shell membrane is moistened with saline to make it transparent, and a small hole made in the chorioallantois over the embryo with fine-tipped forceps such as iris forceps, taking care not to rupture large blood vessels. The amnionic membrane is picked up with the forceps and brought up through the hole in the chorioallantois in a tentlike fold. The inoculum is injected with a syringe and 25 g. needle into the fold. The membrane is then released, and will drop back into its original position. The hole is sealed with strips of scotch tape; the gummed surface of this tape is sterile as it is peeled from the roll.

Harvest. The taped shell down to the edge of the air sac is cut off and the exposed shell and chorioallantoic membranes removed with sterile scissors. The allantoic fluid is removed with a pipette. The amnionic membrane is then picked up with sterile forceps and the fluid inside, containing the virus, is removed with a capillary pipette. If there is very little fluid, the sac can be washed out with a suitable diluent. If desired, the embryo may then be removed and appropriate tissues harvested.

Allantoic Cavity. ***Inoculation.*** The egg is candled, the edge of the air sac outlined, and a line intersecting the edge made at a position where there are no large blood vessels. A small hole is drilled on this intersecting line about 3 mm. above the edge of the air sac. The inoculum, usually 0.2 ml., is introduced by means of a syringe and 25 g. needle, which is inserted through the hole to a depth of about 1 cm. in a line parallel to, but not touching, the shell. The hole is disinfected and sealed with paraffin, collodion, or nail lacquer.

Harvest. The eggs are chilled overnight at 4° C., or for a shorter time in a freezer, to prevent bleeding. After the shell over the air sac is disinfected, it is removed and the exposed shell membrane over the chorioallantoic membrane carefully stripped away with sterile forceps. In the chorioallantoic membrane, away from major blood vessels, a hole is made with a sterile applicator stick, which is then used to hold the embryo and membranes away from the pipette used to remove the allantoic fluid containing the virus.

Chorioallantoic Membrane. ***Inoculation.*** The egg is placed horizontally on a tray, and a new air sac made by dropping the chorioallantoic membrane as follows: After disinfection of the site, a small hole is made in the center of the large end of the egg over the air space. A second hole is drilled, or punched with a dull punch, collared to prevent penetration of more than 0.8 mm., in the center of the uppermost portion of the egg. It is essential that neither the shell membrane nor the chorioallantoic membrane be damaged in this process. A drop of saline is placed over the hole, and the shell membrane carefully broken by means of a sterile penpoint with a small ball tip or slightly upturned tip; the penpoint is inserted through the drop of saline between the shell and the shell membrane, and then slightly depressed and removed. This usually produces a very small rent

in the shell membrane. Then slight suction with a rubber bulb over the hole in the large end of the egg will result in the chorioallantoic membrane's dropping away from the shell. The inoculum is introduced through the hole above the dropped membrane with a syringe and 25 g. needle. The hole is then sealed with scotch tape.

Harvest. The shell above the dropped membrane is disinfected and removed with sterile scissors to expose the membrane. The chorioallantoic membrane is cut out and placed in a sterile petri dish, and spread out and examined for lesions before weighing and grinding. After grinding in a sterile mortar with a small amount of sterile alundum, the appropriate diluent is added, mixed well with the ground tissue, and the suspension centrifuged to remove debris. The supernatant fluid contains the virus.

TISSUE CULTURE

Although animal cells and tissues have been grown in culture for many years, it was not until the antibiotics became available to control the vexatious problem of contamination that successful culture could be carried out generally. As now practiced for the propagation of viruses, tissue culture can be carried out in almost any microbiological laboratory provided the necessary attention is given to exceptional cleanliness of glassware and avoidance of bacterial and fungal contamination. The former includes washing glassware in special detergents and rinsing it in double-distilled or distilled and de-ionized water, while the latter involves use of a hood or small cubicle in the laboratory away from drafts, together with antibiotics in the salt and nutrient solutions.

Culture methods in which the cells are grown on the surface of glass test tubes or bottles are the most frequently employed, and will be described briefly here in terms of examples. Details of these and other tissue culture methods may be found elsewhere.[7, 8] The solutions required are buffered saline and balanced salt solutions, and growth and maintenance mediums.

Salt Solutions. One of the most commonly used buffered saline solutions is the phosphate-buffered saline (PBS) of Dulbecco and Vogt. It has the following composition:

(1)	$NaCl$	8.0 gm.
	KCl	0.2 gm.
	Na_2HPO_4	1.15 gm.
	KH_2PO_4	0.2 gm.
	double-distilled water	800 ml.
(2)	$CaCl_2$	0.1 gm.
	double-distilled water	100 ml.
(3)	$MgCl_2 \cdot 6\ H_2O$	0.1 gm.
	double-distilled water	100 ml.

The three solutions are sterilized separately by autoclaving, and mixed when cool.

The balanced salt solutions (BSS) usually used are Hank's or Earle's; the composition of these is given in the accompanying table. Usually phenol red in a concentration of 1 to 5 mg. per cent is added as an acid-base indicator. The BSS are bicarbonate buffered, and therefore must be sterilized by pressure filtration to avoid loss of carbon dioxide. It follows also that culture tubes and bottles must be tightly stoppered to maintain acid-base equilibrium, and when tissues are grown in petri dishes, an atmosphere containing 5 per cent carbon dioxide is required.

Balanced Salt Solutions
(grams per liter)

	EARLE'S	HANK'S
$NaCl$	6.80	8.00
KCl	0.40	0.40
$CaCl_2$	0.20	0.14
$MgSO_4.7\ H_2O$	0.20	0.20
$Na_2HPO_4.12\ H_2O$	—	0.12
$NaH_2PO_4.H_2O$	0.14	—
KH_2PO_4	—	0.06
$NaHCO_3$	2.20	0.35
glucose	1.00	1.00

Culture Mediums. The culture mediums may resemble bacteriologic mediums as in the case of 0.5 per cent lactalbumin hydrolysate in Hank's or Earle's BSS. Other basal mediums are chemically defined, such as Medium 199 of Morgan, Morton and Parker, and Eagle's basal medium. There are many such basal mediums for tissue culture.

Growth mediums. To support the growth of tissues, such basal mediums require supplemental serum, usually to 10 per cent, the species of serum de-

pending upon the kind of cells being cultured.

Maintenance mediums. When the tissue culture is fully developed, *e.g.*, a continuous sheet of cells covers the glass surface of the kind of culture described here, the growth medium is removed and a maintenance medium substituted during the period of viral replication. In maintenance mediums, the serum added to the basal medium is reduced in amount, or even omitted in some cases, the precise conditions varying with the kind of culture.*

To all of these solutions and culture mediums antibiotics must be added to prevent microbial contamination. These are: penicillin G, 100 units per ml.; streptomycin, 100 μg. per ml.; and Fungizone, 1 μg. per ml.

Preparation of Tissues. Two types of tissue cultures are most often used in virus studies, *viz.*, primary cultures which are cultures of tissue or organ explants that tend to die out on subculture and are necessarily short term, and cultures of continuous cell lines which can be carried indefinitely by serial subculture. Here monkey kidney tissue culture will be taken as an example of a primary culture, and that of the HeLa cell as one of the many cell lines.**

Monkey kidney tissue culture. The capsule, connective tissue and blood vessels in the pelvis are cut away from the kidney and discarded. With sharp scissors or two scalpels the tissue is cut into small pieces about 2 to 5 mm. in size. During this mincing process the tissue is kept moist with PBS, and washed frequently with larger quantities of PBS to remove as much blood as possible.

The minced tissue is transferred to a flask and 50 to 75 ml. of a warm (37° C.) 0.25 per cent solution of trypsin in PBS without calcium, and magnesium (*i.e.*, solution (1) above), is added. The flask is incubated at 37° C. for 15 minutes, the supernatant trypsin poured off and discarded, and fresh trypsin is added, together with a teflon-covered stirring bar. The flask is placed on a magnetic stirrer at room temperature, and the tissue suspension stirred for 10 minutes at a speed sufficient to keep the fragments of tissue in constant motion without foaming. The supernatant trypsin, which now contains kidney cells, is decanted, fresh warm trypsin solution is added, and the process repeated.

Meanwhile the supernatant suspension of cells in trypsin is centrifuged at 700 rpm for 10 minutes to pack the cells. The supernatant is removed and discarded, and the cells are suspended in about 10 ml. of growth medium (0.5 per cent lactalbumin hydrolysate in Hank's BSS with 5 per cent calf serum). Each supernatant suspension of trypsin-released cells is treated in this way, and when the minced tissue has been completely digested, the suspensions of resuspended cells are pooled and filtered through three layers of gauze into a 250 ml. graduated centrifuge tube. The cell suspension is centrifuged at 700 rpm for 20 minutes, and the packed cell volume read.

The cells are now suspended in growth medium, using 300 ml. of medium for each ml. of packed cells. This suspension is transferred to the culture vessels. When these are test tubes, 16 x 125 mm., 0.5 ml. is added to each tube, and the tubes are incubated in a slanted rack so that the cells grow on the side of the tube. If prescription bottles are used, they must contain cell suspension to cover the flat side not more than 5 mm. deep. The uppermost side of the tube or bottle is marked so that, after examination, it can be returned to the incubator in the same position.

Observation of the progress of these cultures can be made with an ordinary compound microscope, using the 10× objective. The tubes or bottles are placed on the microscope stage with the cell layer uppermost. Since this drains the medium from the cells, prolonged observation should be avoided. When the desired cell population is reached, usually after five to seven days incubation at 37° C., the cultures are washed with warm Hank's BSS, and maintenance medium added, 1 ml. to each tube or corresponding amounts to bottle cultures. The maintenance medium, such as Medium 199 without serum, should be one that favors the growth of the virus under study. These cultures are now ready for inoculation with virus, or bottle cultures can be trypsinized as described below for HeLa cell cultures and more cultures prepared; these are often called secondary cultures.

HeLa cell culture. Stock cultures of HeLa cells are usually carried in prescription bottles and transfers made at five to seven day intervals when the cells have formed a confluent sheet on the side of the bottle. Cultures should not be kept longer than seven days because the cell population becomes too large, exhausting the medium and declining in viability.

For subcultures, the growth medium is removed and discarded, the cells washed with two changes of warm Hank's BSS, and sufficient 0.1 per cent trypsin in PBS without calcium or magnesium to cover the cells is added. After

* Many of the solutions and mediums used for tissue culture can be purchased as sterile stock solutions to be diluted in double-distilled water, or complete and ready to use. Sterile serums of many species are also available commercially.

** Such cell lines, and some primary tissue cultures such as monkey kidney, can be obtained commercially.

incubation at 37° C. for about 5 minutes, the cells float into suspension on slight agitation of the bottle. The cells are sedimented by centrifugation at 700 rpm for 10 minutes, the supernatant trypsin solution is discarded, and the cells are suspended in Eagle's medium, containing 10 per cent human serum by repeated pipetting. The cell suspension is counted in a hemocytometer, and diluted appropriately for use as an inoculum. Cultures in 16 x 125 mm. test tubes are inoculated with 50,000 cells in 1 ml., and 8 oz. prescription bottles, used for stock cultures, are seeded with about 10^6 cells.

For virus propagation, the freshly seeded cultures are incubated until sufficient cell growth has occurred, usually about four days. The growth medium is discarded and the cells washed three times with warm Hank's BSS, and maintenance medium added. The medium may be Eagle's medium with 5 per cent chicken serum, or another of the mediums devised for this cell line which is best suited for growth of the virus.

IMMUNOLOGICAL METHODS

The immunological or serological reactions serve to differentiate bacteria on the basis of their constituent antigens. Such immunological differences are usually, though not always, subordinate to the biochemical differentiation of species, and in general serve to subdivide species into immunological types or varieties. The immunological identification of an unknown microorganism is of considerable diagnostic utility and is frequently of very great value in epidemiological studies in tracing the source and dissemination of infection. Furthermore, serum may be tested against a known antigen to establish the presence of the homologous antibody and thus by inference the presence, immediate or past, of the microorganism or its products in the host, and therefore serves as an indirect diagnostic procedure. The specific neutralization of toxin by antitoxin *in vivo* serves to measure antibody in serum or the circulating antibody in the living host, and in the latter is a measure of antitoxic immunity. Of the *in vitro* immunological reactions, those of agglutination of a bacterial or other discrete antigen, precipitation of a soluble antigen, and complement fixation in the presence of soluble or discrete antigen are of general utility.

Titration of Diphtheria Antitoxin by the Römer Method. The intradermal inoculation of diphtheria toxin in the rabbit produces local erythema, and central necrosis with larger doses, which may be used in the assay of toxin, and of toxin in the presence of antitoxin. The end point ordinarily taken is the smallest amount of toxin that, in a dose of 0.2 ml., will produce a zone of erythema 10 mm. in diameter 48 hours after inoculation. This is designated the minimal reacting dose or MRD, and the activity of a toxin may be measured as MRD per ml.

Such a titration measures the potency or toxicity of the toxin preparation, but not its combining power with respect to antitoxin. An expression of the activity of the toxin in the presence of antitoxin is the definition of the Lr dose. This is that amount of toxin which, when mixed with 1 unit of standard antitoxin, contains 1 MRD of unneutralized toxin. It will be clear that a toxin so standardized with respect to standard antitoxin can then be used to titrate an unknown antitoxin by using a constant amount of toxin mixed with serial dilutions of the unknown antitoxin and testing of the mixtures for activity.

One application of this technique is the titration of the antitoxin content of the serum of persons, immunized, convalescent, or normal. This will be considered here since it illustrates all of the principles involved. In studies on human immunity the usual procedure is to titrate the serum against an amount of toxin which will just give the end point reaction when mixed with 0.002 units of standard antitoxin; this is commonly spoken of as titration at the 1/500th level.

Standardization of toxin at the 1/500th level. Toxins vary widely in potency and combining power, and no precise quantitative procedure may be given. The general model of the titration is as follows:

(1) Assume that a toxin whose L+ dose is known is available. Then the following preliminary titrations can be carried out:

(*a*) The standard antitoxin is diluted so that it contains 1 unit per ml.

(*b*) The toxin is diluted so that it contains 1 L+ dose per ml.

(*c*) The toxin is titrated in dilutions of 1:100, 1:200, 1:300, up to 1:1000 against constant amounts of antitoxin, *viz.*, 1 ml. of diluted antitoxin is mixed with 1 ml. of each toxin dilution, incubated as indicated below, 0.2 ml. of each inoculated

Dilutions of Patient's Serum for Titration of Antitoxin at $\frac{1}{500}$th Level

PATIENT'S SERUM (ml.)	SALINE (ml.)	DILUTION	AMOUNT MIXED WITH 1 ML. OF TOXIN DILUTION (ml.)	FRACTION OF ANTITOXIN UNIT TESTED FOR
1.0	0	0	1	1/500
0.5	0.5	1 in 2	1	1/250
0.2	0.8	1 in 5	1	1/100
0.1	0.9	1 in 10	1	1/50
0.1	1.6	1 in 16.6	1	1/30
0.1	2.4	1 in 25	1	1/20
0.1	4.9	1 in 50	1	1/10
0.1	9.9	1 in 100	1	1/5
0.1	49.9	1 in 500	1	1/1

intradermally in the rabbit, and the reaction read in 48 hours. This titration determines the Lr dose of toxin.

(*d*) Additional preliminary titration indicates the approximate amount of toxin required to just give the skin reaction in the presence of 1/500 unit of antitoxin. A more precise determination is carried out as follows:

(i) Prepare a dilution of the original toxin in sterile broth to contain exactly 100 Lr per ml. This is the so-called stock solution and is relatively stable if prepared from stabilized (aged) toxin.

(ii) Dilute 1 ml. of the stock solution to toxin with 99 ml. of buffer solution of give a solution containing 1 Lr per ml.

(iii) Prepare serial dilutions at intervals of 5 within the limits indicated by the above preliminary titration. Thus, if the end point is indicated as lying between 1:40 and 1:80, prepare 1:40, 1:45, 1:50, up to 1:80.

(iv) Mix 1 ml. of each dilution of toxin with 1 ml. of standard antitoxin diluted to contain 0.002 unit per ml. Incubate the mixtures for 1 hour at 37° C. and leave in the refrigerator overnight to allow ample time for the toxin-antitoxin reaction.

(v) Inject 0.2 ml. of each dilution mixture intradermally into the shaven skin of a rabbit (as many as 40 such inoculations may be made in a single animal without ill effects), and identify the sites of inoculation with dye.

(vi) Read at 48 hours, the end point being that dilution of toxin which produces a zone of erythema nearest to 10 mm. in diameter.

Titration of antitoxin at the 1/500th level. For the titration of antitoxin in serum proceed as follows:

(1) Dilute the stock solution of toxin as indicated in the above standardization, *i.e.*, so that 1 ml. contains sufficient toxin to give the skin reaction when mixed with 1 ml. of antitoxin containing 0.002 unit per ml. and inoculated in amounts of 0.2 ml.

(2) Prepare dilutions of patient's serum, say, for illustrative purposes, of 1:10 and 1:500.

(3) Mix 1 ml. of undiluted serum and 1 ml. of each of the dilutions with 1 ml. each of the diluted toxin, and in addition set up a control which contains 1 ml. of the diluted toxin and 1 ml. of standard antitoxin diluted to contain 0.002 unit.

(4) Incubate, inoculate, and read as above.

Suppose the toxin mixed with the 1:500 and 1:10 dilutions of serum gives a skin reaction, but that mixed with the undiluted serum does not. It follows that the undiluted serum contains more than 1/500 unit of antitoxin per ml., but it contains less than 1/50 unit per ml. since it is not neutralized by the 1:10 dilution of serum. By varying the serum dilution, any fraction of a unit may be determined as indicated in the accompanying table.

The Precipitin Reaction. The precipitin reaction is the specific formation of a precipitate when a soluble antigen and its homologous antiserum are mixed. It is used for the identification of soluble antigens of bacterial extracts, such as pneumococcus polysaccharide and the polysaccharide and protein antigens of the streptococci, and is used for the immunological typing of a number of kinds of bacteria. It is also used for the identification of other protein antigens as, for instance, the identification of bloodstains in forensic medicine.

In the precipitin test antiserum is used undiluted, or in very low dilution that avoids undesirable cross reactions, and is admixed with successive dilutions of the antigen. The titer of the antiserum is expressed as the highest dilution of antigen with which precipitation occurs. It is one of the most delicate and sensitive of the serological reactions; antiserums which will precipitate

an antigen such as pneumococcus polysaccharide in dilutions as high as 1:1 to 1:5 million are not uncommon, and titers to serum antigens are frequently 1:10,000 to 1:100,000. The test is carried out as follows:

(*1*) ***Preparation of the antigen dilutions***

(a) Set up a series of small test tubes, such as Wassermann tubes, each with 0.5 ml. of saline solution.

(b) To the first tube add 0.5 ml. of the antigen solution, mix well by blowing in and out of the pipette several times, and transfer 0.5 ml. of this solution to the second tube. The procedure is continued through as many tubes as the titer, or expected titer, of the serum indicates to give a series of dilutions of the antigen to 2^n. If the original antigen solution is not diluted, these dilutions will be 1:2, 1:4, 1:8, etc., but it is more convenient to prepare a 1:100 dilution of the original antigen to begin with, thus giving dilutions of 1:100, 1:200, 1:400, up to 1:25,600, etc.

(*2*) ***Procedure of the test***

(a) Distribute the antiserum in amounts of 0.1 ml. in a series of 5 x 50 mm. test tubes, as many as the dilution range to be observed requires.

(b) Carefully layer 0.1 ml. of the antigen dilutions onto the antiserum, each of the serial dilutions in successive tubes, so that the juncture between the two is clear and sharp.

(c) Incubate at 37° C. for 2 hours, examining at 30-minute intervals for the formation of precipitate at the juncture of antiserum and antigen. This is the *precipitin ring test.*

(d) Shake the tubes to mix thoroughly the antiserum and antigen and store overnight in the refrigerator.

(e) Next day the precipitate will have settled out and can be read by gentle agitation of the tubes.

(f) It is essential that controls of antigen plus saline, and antiserum plus saline, be included, and these should show no precipitate.

The precipitin test may also be carried out in capillary tubes to conserve antiserum, and to utilize the relatively small quantities of antigen available in bacterial extracts as in the typing of streptococci.

Flocculation Tests. The so-called flocculation tests developed for the serological diagnosis of syphilis are related in a sense to the precipitin reaction, and the Kahn test is typical of these. The Kahn antigen is a lipid fraction of heart muscle soluble in alcohol but insoluble in acetone, and contains added cholesterol. When this antigen is mixed with saline in certain proportions floccules appear, and the antigen is titrated to determine the smallest amount of saline which, when added to 1.0 ml. of antigen, produces aggregates which completely disperse upon the addition of more saline. Syphilitic serum has the property of preventing this dispersion. The test is based on the mixture of constant amounts of serum with varying concentra-

Protocol for the Precipitin Titration

TUBE NUMBER	ANTISERUM (ml.)	ANTIGEN	
		DILUTION	AMOUNT (ml.)
1	0.1	1:100	0.1
2	0.1	1:200	0.1
3	0.1	1:400	0.1
4	0.1	1:800	0.1
5	0.1	1:1600	0.1
6	0.1	1:3200	0.1
7	0.1	1:6400	0.1
8	0.1	1:12,800	0.1
9	0.1	1:25,600	0.1
antiserum control	0.1	saline solution	0.1
antigen control	saline solution 0.1	undiluted antigen	0.1

Protocol for the Kahn Flocculation Test

TUBE NUMBER	ANTIGEN (ml.)	SERUM (ml.)	SERUM: ANTIGEN RATIO		SALINE (ml.)
1	0.05	0.15	3:1	Shake vigorously for 2 minutes	0.5
2	0.025	0.15	6:1		0.5
3	0.0125	0.15	12:1		0.5

tions of antigen which are thoroughly mixed by shaking in a shaking machine, and the addition of saline. The test is set up according to the accompanying protocol and read immediately and again 10 minutes after the addition of saline. A positive reaction is indicated by the presence of floccules in the mixture.

Agglutination. Bacteria, and other particulate antigens, aggregate in clumps in the presence of homologous antiserum and are said to be agglutinated. The agglutination reaction is useful in the serological identification of bacteria, especially those of the group of enteric bacilli such as the Salmonella and dysentery bacilli. It is also the immunological reaction made use of, in conjunction with agglutinin absorption, in the antigenic analysis of bacteria.

The agglutination reaction differs from the precipitin reaction in that the antiserum rather than the antigen is diluted, and the antibody titer of the antiserum is expressed as the highest dilution in which a constant amount of bacterial antigen is agglutinated. Agglutinating antiserums commonly show titers ranging from 1:1000 to 1:10,000 and occasionally may reach titers as high as 1:50,000 to 1:100,000, though this is rare. The agglutination of bacteria may be observed either microscopically or macroscopically; the former is generally used for typing purposes, the latter in the titration of antibody.

The microscopic agglutination test. In the microscopic agglutination test either only a few, *e.g.*, two or three, dilutions of antiserum are used, or more commonly, a single dilution is used which preliminary titration has indicated will produce a rapid and complete agglutination with minimal cross reactions. This last allows the use of a single rapid test for typing and certain diagnostic purposes. The test is carried out as follows:

(1) Place a loopful of saline solution on a clean cover slip, and with the straight inoculating wire add a small amount of bacterial growth. The suspension should be faintly turbid, and when this point is reached, the needle is flamed and then used to stir the drop to a homogeneous turbidity.

(2) Place a loopful of diluted antiserum beside the drop of bacterial suspension. Traces of serum remaining on the loop are, of course, removed by flaming.

(3) With the inoculating needle mix the two thoroughly. Note that this produces an additional dilution of the antiserum, and the final dilution is approximately twice the original dilution.

(4) Mount the cover slip on a hollow ground slide with petroleum jelly as in making a hanging drop preparation. No special incubation is necessary, and in a few minutes to an hour at room temperature agglutination occurs.

(5) The preparation may be examined under the low power objective for a curdled appearance or under high power or oil immersion objectives for direct observation of the agglutinated bacteria. False clumping may occur and is distinguished from true agglutination in that in the latter all of the bacteria are gathered in large clumps with none about the edges of the drop, but false clumping occurs in small aggregates about foreign particles and around the edge of the drop.

Macroscopic agglutination. The titration of agglutinin is carried out in a manner similar to the titration of precipitin except, as noted above, with varying amounts of serum in the presence of a constant amount of antigen.

(1) *Preparation of Antigen*

(a) Inoculate an agar slant culture with the bacteria to be used as antigen, spreading the inoculum over the entire surface of the slant.

(b) After 18 hours' incubation run about 2 ml. of saline solution on the agar slant culture. The saline may contain 0.5 per cent formalin if a formalinized antigen is to be used.

(c) Rub up the bacterial growth with an inoculating needle or loop to give a uniform suspension of the bacteria in the saline.

Protocol of Agglutinin Titration

	SERUM DILUTION			AGGLUTININ TITRATION				
	Serum (ml.)	Saline (ml.)	Dilution	Tube Number	Saline (ml.)	Serum (ml.)	Antigen (ml.)	Final Dilution
A	0.1 of undiluted serum	4.9	1:50	1	0	0.50	0.5	1:100
				2	0.25	0.25	0.5	1:200
				3	0.40	0.10	0.5	1:500
				4	0	0.50	0.5	1:1000
B	0.5 of dilution A	4.5	1:500	5	0.25	0.25	0.5	1:2000
				6	0.40	0.10	0.5	1:5000
	0	0		control	0.50	0	0.5	

(d) With flamed forceps remove a small piece of cotton from the bottom (sterile) part of the cotton plug of the culture tube and drop it into the saline suspension.

(e) With a sterile pipette push the piece of cotton to the bottom of the saline, taking care to leave the cotton over the pipette opening.

(f) Draw up the bacterial suspension into the pipette through the cotton, which serves to filter out coarse particles, and add this suspension slowly to the proper turbidity to whatever volume of saline solution is required for the titrations to be undertaken. (A single agar slant culture will make 100 to 300 ml. of antigen, depending on the amount of bacterial growth and the density of the final antigen.) The turbidity of the final antigen should be such that, when diluted with an equal volume of saline, it will be lightly turbid in the test tubes used for the titration. The precise turbidity is dependent upon the kind of antigen and antibody and on the personal preference of the worker.

(2) *Preparation of Serum Dilutions*

(a) Serum may be diluted in essentially the same manner as antigen is diluted in the precipitin test, and the dilution is carried out in 10 x 75 mm. test tubes used for the titration of agglutinin since the antigen is added directly to the diluted serum. A common procedure is to set up a series of saline blanks, the first containing 0.9 ml. and the remainder 0.5 ml. The first dilution is 1:10, made by adding 0.1 ml. of serum to the 0.9 ml. blank; the second and subsequent dilutions are made by transferring 0.5 ml. portions of each preceding dilution. This gives dilutions of 2^n, *i.e.*, 1:10, 1:20, 1:40 . . . etc.

(b) The above method is the one of choice when only a very few titrations are to be run but is laborious and time-consuming if there are many titrations. An alternative, more efficient procedure less subject to dilution error arising from carrying over excess serum in the pipette, since so few pipettes are used that a fresh one for each dilution is practical, is shown in the table on page 36. In this method only one direct dilution is made for each three final serum dilutions, and the dilutions closely approximate 2^n.

(3) *Procedure of the Test*

(a) To each of the series of tubes containing 0.5 ml. each of the several serum dilutions, add 0.5 ml. of antigen suspension and mix by gentle agitation. Note that this doubles the serum dilution, and the final dilutions are 1:20, 1:40, 1:80, . . . etc., when made by the first method; the final dilutions in the second method are given in the table.

(b) The method of incubation is variable, depending upon the particular system being titrated, and the following methods are common:

(i) Incubate at 37° C. or 55° C. for 2 hours, read and store overnight at room temperature or in the refrigerator, and read again.
(ii) Incubate at 37° C. overnight and read the following morning.

(c) The agglutinated bacteria form clumps of precipitate in the bottom of the tubes and are read in a cross light to give a Tyndall effect by gently agitating the tubes individually to swirl the agglutinated bacteria up into the supernatant. The character of the agglutinated bacteria differs in different circumstances; the H agglutination of Salmonella, for instance, is flocculent, while the O agglutination is finely granular. Some workers record degrees of agglutination from + or barely perceptible partial agglutination to ++++ of complete agglutination with a clear supernatant, while others simply record the presence or absence of agglutination with a single + or — sign. The agglutinin titer of the antiserum is the highest dilution in which definite agglutination occurs.

Complement Fixation. The complement fixation test is based on the observation that complement, a heat-labile constituent of normal serum, combines with an antigen-antibody complex and is said to be fixed. Since this fixation produces no visible change, a hemolytic system, consisting of sheep erythrocytes and anti–sheep cell hemolysin, is added. If the complement is free, *i.e.*, has not been fixed, and inferentially the original union of antigen and antibody has not occurred, the erythrocyte-hemolysin complex combines with the complement, and visible lysis of the red cells occurs. Conversely, if the erythrocytes are not lysed, the test antigen-antibody system has combined and fixed the complement, leaving none for hemolysis. This complement fixation test is most often applied in the serodiagnosis of syphilis and virus and rickettsial infections. Bacterial antigens fix complement readily.

The following reagents are required:

(1) The antigen-antibody system to be tested, either component of which may be unknown.

(2) Anti-sheep erythrocyte hemolysin, prepared by the immunization of rabbits with sheep red cells.

(3) A 2 per cent (by volume of packed cells following centrifugation) suspension of washed sheep erythrocytes in saline.

(4) A source of complement, practically always fresh guinea pig serum.

Protocol of Hemolysin Titration

TUBE NUMBER	HEMOLYSIN DILUTION (0.5 ml.)	COMPLEMENT 1:30 DILUTION (ml.)	2% SUSPENSION ERYTHROCYTES (ml.)	SALINE (ml.)
1	1:1000	0.3	0.5	1.7
2	1:2000	0.3	0.5	1.7
3	1:3000	0.3	0.5	1.7
4	1:4000	0.3	0.5	1.7
5	1:5000	0.3	0.5	1.7
6	1:6000	0.3	0.5	1.7
7	1:8000	0.3	0.5	1.7
8	1:10,000	0.3	0.5	1.7
control (9)*	1:1000	none	0.5	2.0
control (10)*	none	0.3	0.5	2.2

* both controls must be negative

Protocol of Complement Titration

TUBE NUMBER	1:30 DILUTION COMPLEMENT (ml.)	SALINE (ml.)	HEMOLYSIN 4 UNITS/ML. (ml.)	2% SUSPENSION ERYTHROCYTES (ml.)
1	0.20	1.8	0.5	0.5
2	0.25	1.8	0.5	0.5
3	0.30	1.7	0.5	0.5
4	0.35	1.7	0.5	0.5
5	0.40	1.6	0.5	0.5
6	0.45	1.6	0.5	0.5
7	0.50	1.5	0.5	0.5
control (8)*	0.00	2.5	0.0	0.5

* control must be negative

Titration of reagents. Since the test is necessarily quantitative with respect to the relative proportions of the various components, the activity of these must be titrated prior to the actual test. These preliminary titrations include:

(1) The titration of hemolysin which is carried out by varying the amount of hemolysin in the presence of constant amounts of sheep erythrocytes and complement, the last in amounts more than necessary for complete lysis. This titration is carried out as indicated in the accompanying protocol. The smallest amount, *i.e.*, highest dilution, of hemolysin required to bring about complete lysis is the *hemolytic unit*. In the final test 2 units in a volume of 0.5 ml. are used in each tube and on the basis of this titration the hemolysin is diluted to contain 4 units per ml.

(2) The complement of fresh guinea pig serum is similarly titrated, using variable amounts of complement in combination with constant amounts of erythrocytes and hemolysin as shown in the accompanying protocol, and the unit of complement is defined. The "exact unit" is the smallest amount that gives complete hemolysis, and the next larger amount, *i.e.*, in the adjacent tube, is the "full unit." In the complement fixation test 2 full units in a volume of 1.0 ml. are used in each tube. The reason for the excess amount is that some complementary activity is lost during the time allowed for fixation; even if no fixation occurs, the test is sufficiently sensitive even with this slight excess.

(3) Certain properties of the antigen are pertinent to the test and must also be determined by preliminary titration. These properties include anticomplementary activity, *i.e.*, inhibition of the action of complement, hemolytic activity, and binding power for complement. The titration of these is shown in the accompanying protocol. In general, the binding power of the antigen should be at least 10 times its anticomplementary action, and in the final test not more than one third of the amount of antigen found to be anticomplementary may be used. It is self-evident that the antigen must not lyse red cells in the concentrations used in the test.

Procedure of the test:

(1) Inactivate the test serum, *i.e.*, destroy any traces of complement in it, by heating to 56° C. for 15 to 20 minutes; dilute 1:5 and distribute in three 15 x 100 mm. test tubes

Protocol of Antigen Titration

PROPERTY TESTED	TUBE NUMBER	ANTIGEN* (ml.)	ANTISERUM* 1:25 DILUTION (ml.)	COMPLEMENT 1:10 DILUTION (ml.)	SALINE (ml.)		HEMOLYSIS 4 UNITS/ML. (ml.)	% SUSPENSION ERYTHROCYTES (ml.)
Anticomplementary activity	1	0.5	0	0.3	0.2	37° C. for 1 hour or store in refrigerator overnight	0.5	0.5
	2	0.4	0	0.3	0.3		0.5	0.5
	3	0.3	0	0.3	0.4		0.5	0.5
	4	0.2	0	0.3	0.5		0.5	0.5
	5	0.1	0	0.3	0.6		0.5	0.5
	6	0.05	0	0.3	0.65		0.5	0.5
Hemolytic activity	7	0.5	0	1.0	1.0		0	0.5
	8	0.1	0	0	1.4		0	0.5
Complement-binding power	9	0.5	0.25	0.3	0.05		0.5	0.5
	10	0.25	0.25	0.3	0.2		0.5	0.5
	11	0.1	0.25	0.3	0.35		0.5	0.5
	12	0.075	0.25	0.3	0.375		0.5	0.5
	13	0.05	0.25	0.3	0.4		0.5	0.5
	14	0.025	0.25	0.3	0.425		0.5	0.5
Controls	15†	0	0.25	0.3	0.45		0.5	0.5
	16‡	0	0.25	0	1.75		0	0.5

* The amounts of antigen and antiserum given here are arbitrary and will vary with individual preparations.

† This control should show complete hemolysis.

‡ This control should show no hemolysis.

Protocol of the Complement-Fixation Test

TUBE NUMBER	SERUM DILUTED 1:5 (ml.)	ANTIGEN 20 units/ml. (ml.)	SALINE (ml.)		COMPLEMENT 2 units/ml. (ml.)		HEMOLYSIN 4 units/ml. (ml.)	2% SUSPENSION ERYTHROCYTES (ml.)
1	0.5	0.5	0	Let stand	1.0	37°C. for	0.5	0.5
2	0.25	0.5	0.25	at room	1.0	1 hour, or	0.5	0.5
3	0.125	0.5	0.375	temperature	1.0	refrigerator	0.5	0.5
4 (SC)	0.5	0	0.5	for 10	1.0	overnight	0.5	0.5
5 (AC)	0	0.5	0.5	minutes	1.0	and 5–10	0.5	0.5
6 (HC)	0	0	1.0		1.0	min. 37°	0.5	0.5
7 (EC)	0	0	2.5		0	C.	0	0.5

* Set up as the Kolmer modification of the Wassermann test.

SC serum control—should show complete hemolysis

AC antigen control—should give complete hemolysis

HC hemolytic system control—should give complete hemolysis

EC erythrocyte control—should give no hemolysis

as indicated in the protocol on page 38. Bring the total volume in tubes 2 and 3 to 0.5 ml. by adding 0.25 and 0.375 ml. of saline respectively.

(2) Add 0.5 ml. of antigen, diluted to contain 20 complement-fixing units per ml. to each tube.

(3) Let the mixture stand at room temperature for 10 minutes.

(4) Add 1.0 ml. of complement, diluted to contain 2 full units per ml., to each tube.

(5) The mixture may be incubated in a water bath at 37° C. for 1 hour, or stored in the refrigerator overnight and warmed in the water bath for 10 to 15 minutes before proceeding with the test the following day. This time interval is allowed for fixation of the complement by the antigen- antibody

complex, and the later method is referred to as "icebox fixation."

(6) Add 0.5 ml. of hemolysin diluted to contain 4 hemolytic units per ml. to each tube.

(7) Add 0.5 ml. of a 2 per cent suspension of washed sheep erythrocytes to each tube.

(8) The appropriate controls of the serum, antigen, hemolytic system, and erythrocyte suspension are indicated in the protocol and *must be included.*

(9) Incubate in a water bath at 37° C. for 15 to 60 minutes.

(10) Read the hemolysis as complete (++++), partial (+++, ++, +, ±), or negative.

REFERENCES

1. American Public Health Association: Diagnostic Procedures for Virus and Rickettsial Diseases. American Public Health Association, New York. 2nd ed., 1956.
2. American Public Health Association: Diagnostic Procedures and Reagents. Technics for the Laboratory Diagnosis and Control of the Communicable Diseases. American Public Health Association, New York. 3rd ed., 1950.
3. Beveridge, W. I. B., and Burnett, F. M.: The Cultivation of Viruses and Rickettsiae in the Chick Embryo. Medical Research Council (Great Britain), Spec. Rept. Ser. No. 256. 1946.
4. Chatigny, M. A.: Protection against infection in the microbiological laboratory: Devices and procedures. Adv. Appl. Microbiol., 1961, **3:** 131–192.
5. Conn, H. J.: Biological Stains. Williams and Wilkins, Baltimore. 7th ed., 1961.
6. McNew, G. L.: Dispersion and growth of bacterial cells suspended in agar. Phytopathology, 1938, **28:**387–401.
7. Parker, R. C.: Methods of Tissue Culture. Hoeber, New York. 3rd ed., 1961.
8. Paul, J.: Cell and Tissue Culture. Livingstone, Edinburgh and London. 1959.
9. Society of American Bacteriologists: Manual of Microbiological Methods. McGraw-Hill, New York. 1957.
10. Society for General Microbiology: Constituents of Bacteriological Culture Media. Cambridge University Press. 1956.
11. Simmons, J. S., and Gentzkow, C. J.: Medical and Public Health Laboratory Methods. Lea and Febiger, Philadelphia. 1955.
12. Symposium. The chick embryo in biological research. Ann. N. Y. Acad. Sci., 1952, **55:**37–344.

Chapter 3

THE PHYSICAL AND CHEMICAL STRUCTURE OF MICROORGANISMS

One of the most striking aspects of the morphological consideration of microorganisms is their extremely small size. This order of size is such that they are most conveniently measured in the units of the micron ($\mu = 10^{-3}$ millimeters) and the millimicron ($m\mu = 10^{-6}$ millimeters). The bacteria proper, those capable of independent metabolic existence in that they are cultivable on lifeless nutrient mediums, range in size from that of the large bacilli,* such as *Bacillus anthracis*, with dimensions of 1.0 to 1.3 x 3 to 10 μ, to minute forms such as *Pasteurella tularensis* measuring 0.2 x 0.2 to 0.7 μ and microorganisms of the pleuropneumonia group in which viable elements as small as 175 $m\mu$ are demonstrable.

The rickettsiae and viruses, characterized by their inability to proliferate apart from a living host cell, form a continuous series of even smaller forms, running through and beyond the limits of optical resolution, commonly taken to be 200 $m\mu$ but in practice about 250 $m\mu$. The rickettsiae resemble the small bacteria, having dimensions of 0.2 x 0.5 to 1.5 μ, and the pox viruses, whose elements measure 200 to 350 $m\mu$, also overlap the smallest bacteria. From this maximum, the viral agents range down to nearly macromolecular dimensions such as 25 $m\mu$ for poliomyelitis virus and 22 $m\mu$ for foot-and-mouth disease virus, while the egg albumin molecule, for example, measures 2.5 x 10 $m\mu$. The relative size of representative microorganisms is illustrated in Figure 3.

The range in size among individual bacterial cells of the same species differs from one species to another, being greatest in the bacillary forms and least in the spherical types. Excluding filamentous and swollen forms that may be produced under appropriate conditions and are usually regarded as abnormal, the maximum difference in size between mature individual cells is about fourfold to fivefold.

The structure and physiological processes of a living organism are intimately associated with, and in fact are a function of, its size.[135] While substantially the same mechanisms are functional in terrestrial mammals from the elephant (4000 kg.) to the pigmy shrew (4 gm.), a mammal smaller than the very small rodents cannot exist because it could not assimilate and metabolize nutriment rapidly enough to supply the energy it requires. Similarly, the rate of oxygen

* Excluding the often quoted but exceptional case of *Bacillus bütschlii*, found in the cockroach intestine, and reported to be as large as 0.6 x 80 μ.

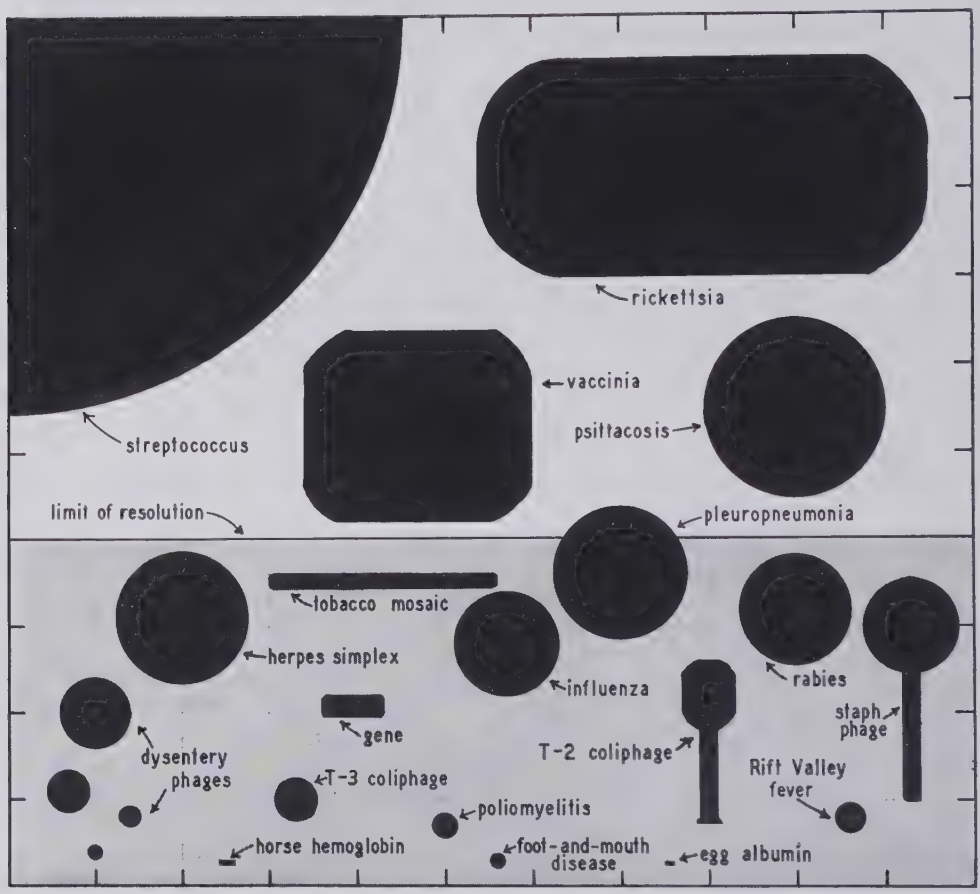


Figure 3. The relative size of representative microorganisms. The pips are at intervals corresponding to 100μ, and the small squares of the grid to 10 mμ. The microorganisms in the shaded portion are those whose size is below the limits of resolution of the light microscope.

diffusion in insect tracheas limits the size range of organisms using this respiratory mechanism between a maximum represented by some tropical forms 5 to 8 cm. in length and the minimum of small mites 0.2 mm. long.

The lymphocyte, one of the smallest mammalian cells, with a diameter of 10 μ and a volume of 524 μ^3, is many times larger than *B. anthracis* with a maximum volume of 22 μ^3, and the minimum volume of 0.004 μ^3 of *Past. tularensis*. The last appears to represent the minimum size that allows the degree of organization necessary to independent metabolism in that the smaller agents, the viruses, are obligate intracellular parasites.

The size range of the obligate parasites overlaps that of the metabolically independent microorganisms in that the larger forms, such as *Coxiella burnetii* having a volume of 0.02 μ^3 and the largest viruses, such as vaccinia virus with a volume of 0.008 μ^3, are larger than *Past. tularensis* and other extremely small bacteria. But smaller forms, running in continuous series to the apparent minimum of the volume of 0.00005 μ^3 of foot-and-mouth disease virus, can at best contain only relatively few protein molecules whose orientation is hardly comparable to cell structure as ordinarily understood.

While metabolic processes on a molecular level need not differ significantly between large and small physiological units, it may be anticipated that the relative lack of cellular structure may result in unique characteristics of the minute forms. This has proved to be true, in part because these minute forms

do not appear to be closely restricted to the hereditary mechanisms, processes of proliferation, etc., to which the more elaborately organized unicellular and multicellular organisms are limited, and in part because of the special circumstances of their macromolecular environment. The uniqueness of the microorganisms is more apparent than real, then, in that their unusual properties and behavior represent an extension of, rather than a contrast with, the patterns of basic biological phenomena.

MICROSCOPY[28]

A consideration of the technical limitations and advantages of the various methods available for the demonstration of the shape and structure of microorganisms is an essential preliminary to a consideration of the morphology of these forms. In general, these methods are of two kinds, *viz.*, those utilizing light, both visible and ultraviolet, and that making use of a focused beam of electrons.

The Compound Microscope. With the ordinary microscope, objects are viewed by transmitted light, *i.e.*, the object is interposed between the source of light and the eye or photographic plate. In order to be seen and delineated (resolved) the object must be sufficiently large to cast a shadow, and the critical size is approximately half the wave length of the light used. The highest numerical aperture that is practical in the objective lens is 1.4, and this permits resolution of objects down to the critical size. The limit of resolution is obviously a function of the wave length of light used. Thus in the range of 5000 Å (500 mμ), or blue light, to 5500 Å (550 mμ), or yellow light, the smallest object that can be resolved is a sphere having a diameter of 250 to 275 mμ. Theoretically an object as small as 200 mμ can be resolved using deep blue light at 400 mμ wave length, but the practical limit is less than this.

Resolving power may be appreciably extended by the use of light in the ultraviolet region; assuming a wave length of 250 mμ, a sphere 125 mμ in diameter may be resolved. Quartz lenses are, of course, required.

The Darkfield Microscope. In the darkfield microscope reflected rather than transmitted light is used, taking advantage of the familiar Tyndall effect perhaps most commonly seen in the appearance of particles of dust in a shaft of sunlight in a darkened room. The usual compound microscope may be used by substituting a darkfield condenser, which lights the object from the side, for the ordinary substage condenser. Relatively large amounts of light are required, and oil is placed between the top of the condenser and the slide to reduce loss at these interfaces.

The object appears brilliantly lighted against a dark background. There is an illusion of increased resolution in that extremely small objects appear as bright points of light in contrast with the minute shadows cast in transmitted light, but, in fact, resolution is not increased beyond the critical limits noted above. Thus the darkfield microscope has not contributed appreciably to bacterial cytology other than with regard to certain aspects of the activity of flagella. It has, however, very considerable utility in the demonstration of very slender microorganisms, especially spirochetes, as in exudate from a syphilitic chancre or leptospira in the blood from cases of leptospirosis.

The Phase Microscope.[13, 99] Light passing through an object of different thickness or refractive index from that of the surrounding fluid is scattered, with the result that it is out of phase and reduced in amplitude, *i.e.*, the distance from the crest to the trough of the light wave is reduced. Differences in amplitude are visible and allow the observation of the object as a relatively faint shadow against its background, and bacteria may be so observed in the living unstained state, usually in a wet preparation.

While differences in phase are not ordinarily visible, they may be made so by treating the light scattered by the

object and that coming through the surrounding fluid independently so that they interact, either to interfere with one another to darken the image or to reinforce one another to brighten it. This is accomplished with the ordinary microscope by restricting the incident light to an annular beam by means of an annular diaphragm at the lower focal plane of the condenser and the insertion of a corresponding annulus or diffraction plate at the back focal plane of the objective lens. The microscope so modified is a phase microscope and may be either bright or dark phase.

Phase contrast microscopy has been particularly useful in the study of structures in the living cell, unmodified by the artifacts inherent in the processes of fixing and staining. By increasing contrast, it gives greatly improved resolution, for example, allowing the demonstration of bacteria in unstained tissue sections that are not resolved by the bright- or darkfield microscopes because of the relatively great diffraction and reflection of the tissue elements. The phase microscope is, however, subject to the same limits in ultimate resolving power as other forms of the microscope using visible or ultraviolet light.

The Electron Microscope.[25, 148] The electron microscope operates on a different principle from that of the optical microscopes in that a stream of electrons is used rather than a beam of light. The electron beam is focused by magnetic "lenses" and the interposed object intercepts the beam to cast a shadow which is recorded on a photographic plate to give an electron micrograph. The theoretical resolving power of the electron beam is about 100,000 times that of visible light, but this limit is not reached in practice because of defects in the magnetic focusing corresponding to chromatic and spherical aberration in optical systems. Nevertheless, remarkable resolution is possible at 10,000 to 50,000 diameters and, because of the small aperture used, the depth of focus is considerably greater than that obtainable with the 1.4 mm. objective.

The technique of shadow-casting with heavy metals vaporized under high vacuum and deposited at an angle on the preparation was developed by Williams and Wyckoff in 1946. The metal film is opaque to electrons, and the resulting electron micrograph has a brilliantly lighted three-dimensional appearance.

Application of electron microscopy to microorganisms has made it possible to see details of structures lying at the limits of optical resolution and to determine directly the size and shape of small viruses previously studied only by indirect methods such as sedimentation and filtration. The results of electron microscopy must be interpreted with some caution because specimens can be examined only as dead dry material because of the high vacuum required and are subject to the very considerable distortion of drying. Electron microscopy is, then, an extremely valuable adjunct in the study of, for example, cell structures that may also be examined in the living cell by other means, but when the structure can be resolved only in the electron microscope, its size, shape, etc., may be accepted only with reservations.

THE MORPHOLOGY OF BACTERIA[64, 65, 133]

The morphology of bacteria may be considered under two general heads: that of individual cells and groups of cells as observed under high magnifications, and that of bacterial colonies developing on solid mediums and consisting of very large numbers of cells that are visible to the naked eye. In the first instance differences in shape and certain structural details as well as size are characteristic of at least the main groups of bacteria and provide the primary basis for their systematic study. Similarly, the masses of cells represented

in the bacterial colony also have characteristics such as size, shape, consistency, texture, and color that have differential value though not the fundamental significance of cellular morphology.

THE BACTERIAL CELL

Shape. The most obvious difference among bacteria is their shape, and three general morphological types are clearly evident. These are the spherical form or coccus, the rod-shaped form or bacillus, and the spiral forms, the various subtypes of which are the vibrio, spirillum, and spirochete.

Coccus. The spherical bacteria are by far the most homogeneous of the bacteria with respect to size and, in general, have a diameter of 0.8 to 1.0 μ, although smaller varieties, 0.4 to 0.8 μ in diameter, have been described. There is some small deviation from the spherical form among bacteria grouped as cocci. The pneumococcus, for example, has a lanceolate shape, slightly elongated with one end more pointed than the other, and the gonococcus and meningococcus tend to a coffee bean shape. Other slightly elongated forms are referred to as coccobacilli, and the line of demarcation is not precise.

Morphological subtypes of the cocci are differentiated on the basis of the arrangement of the individual cells with respect to one another. This is a consequence of two factors, one the plane or planes in which cell division occurs, and the other the tendency of daughter cells to remain superficially attached or close to one another after division is complete.

Those cocci which separate completely after cell division, regardless of the plane of division, appear singly, and scattered at random over the microscopic field are called *micrococcus.* When there is a slight tendency for daughter cells to remain attached, and cell division occurs in only one plane, the cocci tend to occur as pairs, *diplococcus,* but the paired cells are intermingled with individual cells. Some kinds of diplococci are slightly elongated and are paired with the long axes parallel. In the case of the coffee bean–shaped gonococcus and meningococcus noted before, the paired cells have the convex sides adjacent. When the tendency to remain attached is more marked, the result is chains of cells, *streptococcus,* consisting

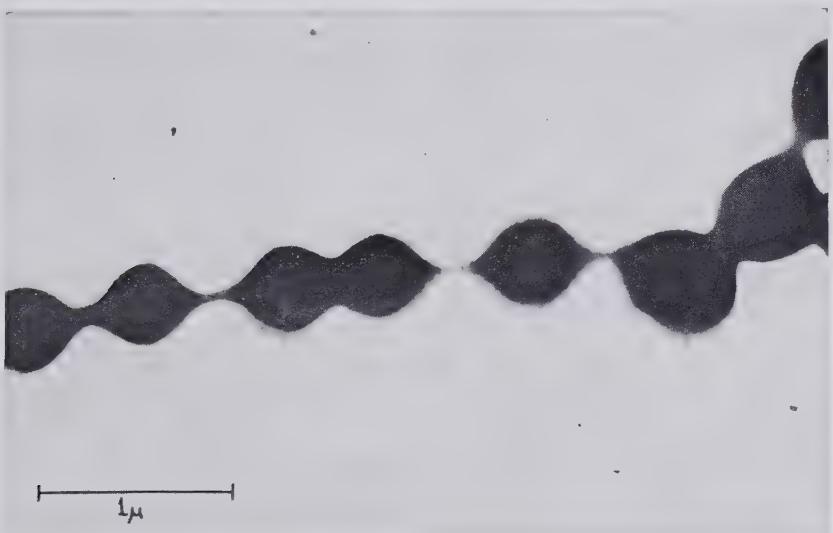


Figure 4. Electron micrograph of *Streptococcus pyogenes.* Note the manner in which the individual cells remain attached to one another following fission and the tendency of the chains to be made up of attached pairs of cells. (Courtesy of Drs. S. Mudd and D. Lackman, and the Journal of Bacteriology.)

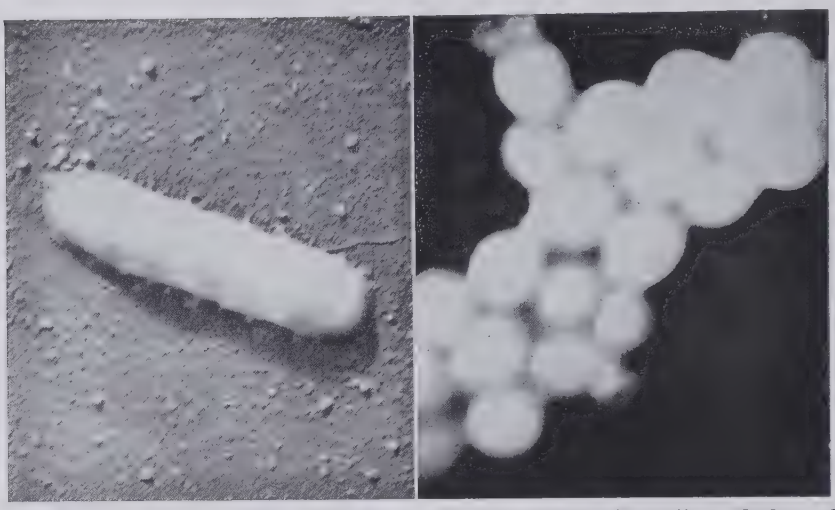


Figure 5. Electron micrographs of gold-shadow-cast preparations of *Bacillus subtilis* and *Staphylococcus aureus*. The shadow-cast preparation illustrates particularly well the occurrence of staphylococci in clusters of attached cells. The cap at the ends of the bacillus and a single flagellum are shown. (Lilly Research Laboratories.)

of four to ten or a dozen cells that often have the appearance of diplococci attached end to end.

When there is a marked tendency of daughter cells to remain adjacent to one another coupled with cell division in two or more planes, the cocci occur as irregular groups, *staphylococcus*, which are often quite large. When observed in the usual stained preparation, staphylococci appear to consist of sheets of cells, *i.e.*, only one cell thick, but on examination in a wet mount are seen to consist of irregular, grape-like clusters. The former would result from cell division occurring in only two planes, and the latter from division in three planes, but it is probable that the sheets of cells are, for the most part, an artifact resulting from the preparation of the smear. When the tendency to remain together is less marked, the cocci are found in tetrads or groups of four cells and in cubical packets of eight cells. There is no casual name for these smaller aggregates, but they are characteristic of some genera, such as Sarcina.

Separation of these subtypes of coccus on a morphological basis is not completely sharp. Thus, diplococci occur admixed with single cells, streptococcus chains are interspersed with paired and single cells, and single and paired cells, as well as an occasional chain of four cocci, are found in stained smears of staphylococci. It is obvious that these groupings can be broken up to a greater or lesser extent in preparing the bacterial suspension for examination and that the occurrence of typical groupings is not absolute, but the consequence of only a tendency to remain adjacent. In practice, though, the characteristic morphological groups are usually found without difficulty and are most consistently observed among the staphylococci.

Bacillus. The cocci merge into the rod-shaped bacteria through connecting links such as the lanceolate coccus and coccobacillus noted above. In turn, the rod-shaped or bacillary form is a collective term that embraces a variety of morphological subtypes.

The morphology of individual rod-shaped bacteria differs considerably between genera and even species. Not only is there variation in size, but the shape of the individual cell differs, sometimes markedly. Some are long and slender while others may be short and thick; the sides may be more or less parallel to one another, or the cell may be thicker in the center and taper toward the ends to give the form known as the *fusiform bacillus*. Similarly, the ends of the cells may be square and cut off sharply, or even give

the appearance of a slight concavity, while the ends of other cells are rounded. These variables can occur in a number of combinations to give a considerable heterogeneity in the bacillary form. A given shape is, however, relatively constant within a species under standard conditions of growth, though the length-width ratio may vary, owing in large part to elongation of individual cells prior to cell division.

Like the cocci, some of the bacillary forms assume characteristic groupings, but these are a consequence of postfission movements, for the plane varies rarely if at all from that vertical to the long axis. There is a tendency of the cells to remain attached, or closely adjacent, after cell division in some kinds of bacilli; it is marked in some forms, notably the sporeforming aerobic organisms of the genus Bacillus, and may be enhanced under appropriate conditions of growth. When this occurs, it results in the formation of chains of cells, the *streptobacillus* morphological type.

The postfission movements of bacilli are of two general kinds. The one, known as *slipping,* consists of a sliding movement of the cell against one another with the long axes parallel to give groups of cells with a palisade-like appearance. This appears to be a consequence of space limitations interfering with continued extension of growth in a longitudinal axis.[57] In the other kind of movement, *snapping,* after division the daughter cells bend sharply with respect to one another to an acute angle to give a V-shaped appearance. When this kind of movement is associated with a tendency to remain attached end to end, chains of cells resembling a split-rail fence result.

While to a certain extent characteristic, the groupings assumed by the rod-shaped bacteria have only minor differential significance in contrast to the cocci among which they make possible morphological subtypes of generic status.

The spiral forms. The third main morphological type of bacteria is made up of the spiral forms which may be regarded as bacillary forms twisted in the form of a helix. Again, forms intermediate on a morphological, though not necessarily phylogenetic, basis occur as as the curved rods. These range from the occasionally curved coliform bacilli to those forms whose curvature is sufficiently consistent to have differential significance in the definition of the genus Vibrio. Whether the vibrios are truly transitional forms linking the slender bacilli with the spiral microorganisms is questionable, but their occasional end-to-end grouping in which the direction of curvature of the individual cells alternates superficially resembles the spiral form.

The spiral bacteria, which if straightened out would be long slender bacilli, are of two general kinds, the one in which the spiral is rigid, the genus Spirillum, and the other in which it is flexible. Among the latter, a further differentiation is made on the tightness of the coiling. The tightly coiled forms include several genera which are differentiated by finer morphological criteria. These include differentiation of an outer sheath, a central filament about which the protoplasm is coiled, etc.; such struc-

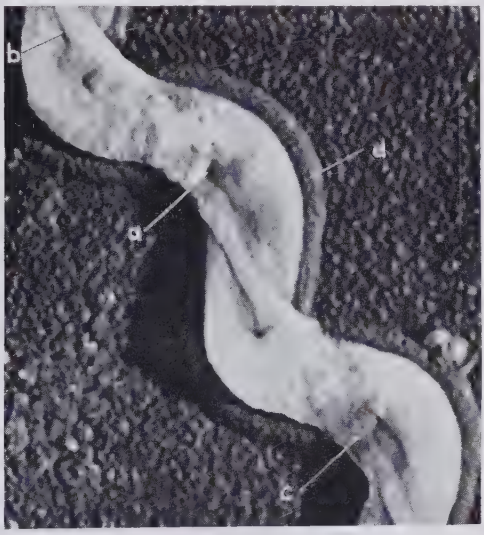


Figure 6. Electron micrograph of a chromium shadow-cast preparation of *Leptospira icterohaemorrhagiae.* The axial filament is separated from the protoplasmic spiral by a narrow space at (a) and is more closely approximated at (b). The sheath is shown at (d). × 125,000. (Simpson and White.[120])

tural details are illustrated in the accompanying electron micrograph.

One of these is the genus Treponema which includes pathogenic forms, notably the causative agent of syphilis and related diseases, together with a wide variety of nonpathogenic but parasitic forms such as those making up a part of the normal flora of the mouth. Still another form, distinguished by sharp, hooklike bends at the ends of the cell, makes up the genus Leptospira, a causative agent of infectious jaundice.

Much less tightly coiled spiral forms which have, in fact, the appearance of extremely long, slender, undulating bacillary forms, make up the third morphological subtype. This includes both parasitic and pathogenic forms, the latter causing the louse- and tick-borne relapsing fevers in man and related diseases in certain fowl, and they are grouped under the genus Borrelia.

Taken together, the spiral forms are casually referred to as the spirochetes. The emphasis on morphological criteria for purposes of differentiation and definition of genera is a consequence of their failure, with the exception of Leptospira, to grow on laboratory mediums and, therefore, a complete ignorance of differential physiological activity that provides much of the basis for the characterization of other bacteria.

Involution Forms. The great majority of bacteria are relatively constant in shape and size in young cultures actively growing under favorable conditions. In older cultures in which many of the cells are dead or dying, the cell structure breaks down and aberrant forms appear. These include balloon-like structures, Y-shaped bacillary forms, cells containing large amounts of granular material, and the like. These are degenerative forms or *involution forms* and presumably result from breakdown of the mechanisms of selective permeability with imbibition of water, autolysis of cell structures by the action of proteolytic and other intracellular enzymes, etc. Some workers have attached significance to bizarre forms found in older cultures, regarding them as representative of an orderly sequence of morphological types indicative of a complex life cycle. This view is discussed elsewhere in connection with microbial genetics and variation.

Aberrant forms of bacteria may also be produced by growing them under somewhat adverse conditions, such as

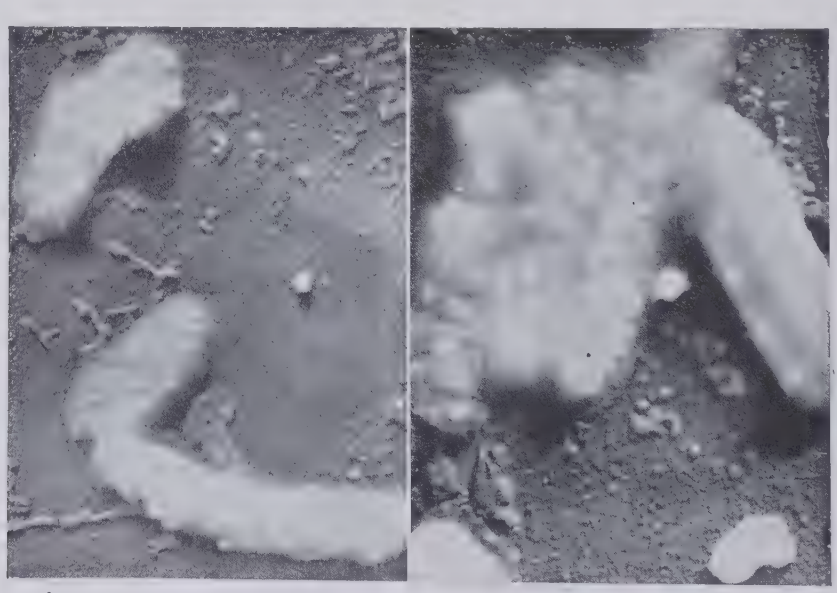


Figure 7. Electron micrographs of gold-shadow-cast *Bacterium coli* from old cultures. One intact cell is present, and the remainder show the granulation of dead and disintegrating cells. (Lilly Research Laboratories.)

higher than optimum temperatures, in the presence of relatively large concentrations of inorganic salts,[51] and sublethal concentrations of antibacterial substances. In the case of the last, the antibacterial activity may be largely growth-inhibiting rather than bactericidal, one result of which may be an inhibition of the processes of cell division rather than of synthesis of cell substance, so that giant cocci, etc., develop in such cultures.

THE STRUCTURE OF BACTERIAL CELLS

The total physiological activity of the bacteria is, in many respects, so closely similar to that of the larger forms, that the assumption of at least some common mechanisms on a molecular or macromolecular level is more than a working hypothesis. While it is obvious from even cursory examination that the bacterial cell is discontinuous[55] and therefore has structure, at the same time considerations of size make it equally clear that its structural elements need not be, and in fact in some instances perhaps cannot be, small scale replicas of those found in larger cells. The problem becomes, then, one of elucidation of the nature of subcellular elements and of their relation to the physiological functions of the cell.

The reference point is necessarily the living cell, and the information obtained by direct observation is subject to the limits imposed by the degree of optical resolution possible with visible, or ultraviolet, light. This basic information is supplemented and complemented by studies on killed cells treated with stains and other cytochemical reagents and/or subjected to observation under the high magnifications obtainable in the electron microscope. Such supplementary information must be treated with due reservation because of artifacts resulting from fixing, drying, and other preparative procedures.

For purposes of discussion the bacterial cell structures may be divided into two groups, those structures which are external to the cell proper and those which occur within the cell.

External Structures

Two kinds of structures are external to the bacterial cells, the flagella or organs of locomotion and the capsular material or slime layer surrounding the cell. Neither is essential to the continued existence of the cell. A bacterium may be deprived of its flagella, as by mechanical shaking, or of its capsule by enzymatic digestion, without inhibition of growth or metabolic functions.

Flagella.[70, 128, 142] The bacterial flagellum is a long filamentous appendage originating in a spherical body, or basal granule, about 100 mμ in diameter and located in or just inside the cell wall. It is not clear whether these bodies correspond to the blepharoplasts of ciliated protozoa, but flagella are distinct from, though analogous to, cilia.

The flagellum is usually 12 to 15 mμ in diameter, without taper, and of variable length, in an extreme range of 1 to 70 μ, but ordinarily 15 to 25 μ. In the living cell it is coiled in the form of a cylindrical helix, but in dried preparations the third dimension is minimized and it appears as a wavy filament. Because of its length the flagellum is demonstrable occasionally in the living cell by phase contrast microscopy and more readily with the darkfield microscope. In mordanted stained preparations, the stain is deposited on the flagellum to make it sufficiently thick that it may be observed with the conventional microscope and is, of course, readily seen in electron micrographs.

Flagella occur most commonly, although not exclusively, among the rod-shaped bacteria. They may be attached either at or near the poles of the cell or distributed over the rest of the cell surface with a tendency for the poles to remain bare, and their location and number are relatively constant within bacterial species. The classification of bacteria with respect to the occurrence of

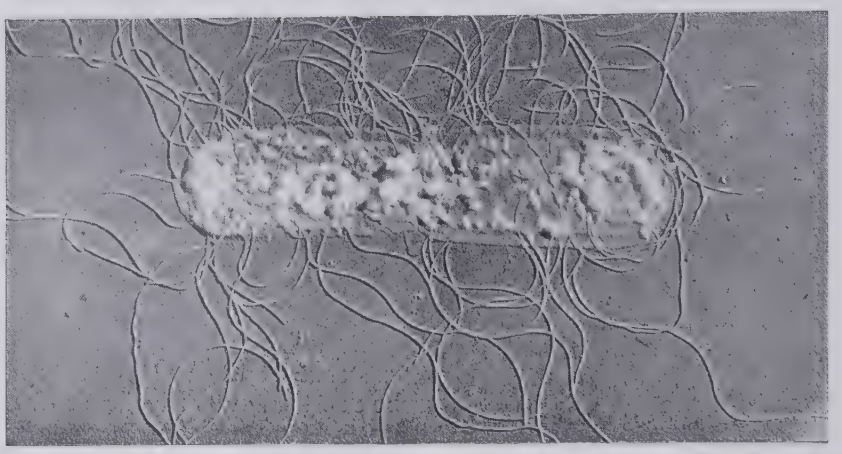


Figure 8. Electron micrograph of shadow-cast preparation showing the flagella of *Proteus vulgaris*. The bacteria were grown to the swarming stage on agar, refrigerated at 5° C. for 16 to 20 hours, floated off in 5 per cent formalin, and washed twice before mounting on collodion. The preparation is very transparent and flattens completely so that certain of the internal structures are demonstrable by shadowing. The origin of the flagella in small, spherical bodies about 100 mμ in diameter and remarkably uniform in size is clearly shown. (van Iterson.)

flagella is the early one of Messea. According to this, the bacteria having a single polar flagellum are designated monotrichous, those having two or more flagella at one end of the cell lophotrichous, those having tufts of flagella at both ends of the cell amphitrichous, and those having flagella, usually 8 to 12, distributed over the cell surface are peritrichous.[70]

The occurrence of flagella on bacteria is associated with motility, *i.e.*, a movement of translation as distinct from brownian movement. While it has been urged by some, especially Pijper,[95] that bacterial flagella are no more than threads and streamers of viscous capsular substance twisted off by a screwlike motion of the cells and are, therefore, artifacts, this view is not generally accepted. The several lines of evidence, such as that of microscopy or immunological character of flagellar substance, all support the view that the flagellum is a true cell structure, or organelle, and is responsible for motility.[129]

The rate of movement of motile bacteria is often surprisingly rapid. For example, the motile enteric bacilli, such as the typhoid, coliform, and related bacteria, have been found to average speeds of 25 to 30 μ per second and the cholera vibrio to move as fast as 55 μ per second.[93] These rates are comparable with automobile speeds of 40 to 80 miles per hour.

The flagellar substance is antigenically distinct from that of the cell, and antibody to it is specific. The function of flagella in motility is seriously interfered with in the presence of specific antibody, and the organisms are immobilized because of agglutination of the flagella.[84] This is the basis of the "immobilization test" which has considerable serodiagnostic utility in diseases such as syphilis.

Flagella also differ in chemical composition from the cell substance proper. They may be separated from the cell by vigorous shaking, following by differential centrifugation, and concentration by high speed centrifugation, salting out, or cold ethanol precipitation. The resulting preparations may be shown by electron micrography to consist of filamentous material, presumably representing fragmented flagella, which is relatively homogeneous on sedimentation and electrophoresis and behaves like a solution of long, thin protein molecules. Its amino acid composition is characteristic, and no histidine, tryptophan, hydroxyproline, or cystine is found in detectable amounts. Since the chemical composition of flagellar protein is substantially the same in

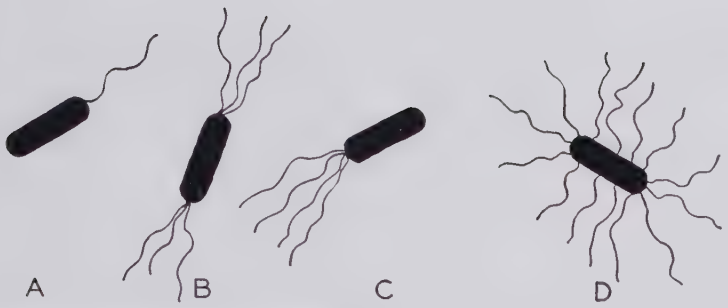


Figure 9. Position of flagella on the bacterial cell. *A*, monotrichous; *B*, amphitrichous; *C*, lophotrichous; *D*, peritrichous.

different genera of bacteria, such as Proteus and Bacillus, it has been suggested that antigenic differences are the result, in part at least, of differences in the sequence of amino acid residues in the peptide chains.

X-ray diffraction studies have shown that the flagellar protein, for which the name flagellin has been suggested, is similar to keratin, myosin, epidermin, and fibrinogen. Two patterns are observed, the one suggesting polypeptide chains folded in the α-configuration, and the other the cross-β-configuration regarded as characteristic of actively contracted muscle. This may be taken to imply that the motion of flagella attached to the living cell is a result of rhythmic interchanges between the two configurations.[9]

The motion of flagella is apparently random; the fibrous internal conducting structures found in ciliates seem to have no counterpart in the motile bacteria, and such a "primitive nervous system" is lacking. The seemingly coordinated motion of flagella is a hydrodynamic phenomenon, occurring when the random flagellar motions of a multiflagellar bacterium coincide by chance to initiate movement of the cell.

The energy expenditure required for motility has been calculated[91] to be about 14 electron volts per second, or, assuming a 25 per cent efficiency, a total output of 56 electron volts per second. Further, the analogy between muscle fibers and the observed contraction of isolated flagella in the presence of ATP has been taken to suggest that the source of energy is that liberated in the metabolism of energy-rich phosphate bonds, with about 150 bonds reacting per second. Since each flagellum flicks 10 to 20 times per second, and assuming 10 to 20 flagella on a peritrichous bacterium, it is possible that each flick is the consequence of a discrete metabolic event, such as the breakdown of an energy-rich phosphate bond.

Fimbriae. In a study of bacterial flagellation, Houwink and van Iterson[61] described minute filamentous appendages on some kinds of flagellated bacteria which have subsequently been referred to as pili, bristles, filaments, and fimbriae; the last appears to have gained some general acceptance. Studies by others[22, 35, 36, 45] have shown that fimbriae are less rigid than flagella, and considerably smaller, 10 $\times$ 300 to 1000 mμ, 100 to 250 per bacterial cell. In the case of dysentery bacilli they appear to facilitate adherence of the bacteria to the intestinal mucosa and, in the Flexner dysentery bacilli, contain an antigen common to the several serotypes (Chap. 22). Not all flagellated bacteria have fimbriae, and as yet their significance is uncertain.

Capsules.[64, 136, 137] Many bacteria, probably all if grown under appropriate environmental conditions, are surrounded by a layer of gelatinous, poorly defined, and poorly staining material that has been variously termed the capsule, slime layer, or envelope. While there is a tendency to apply one term to one kind of bacterium and another to another kind, *viz.*, the capsule of the pneumococcus, the slime layer of Leuconostoc, and the envelope of the plague

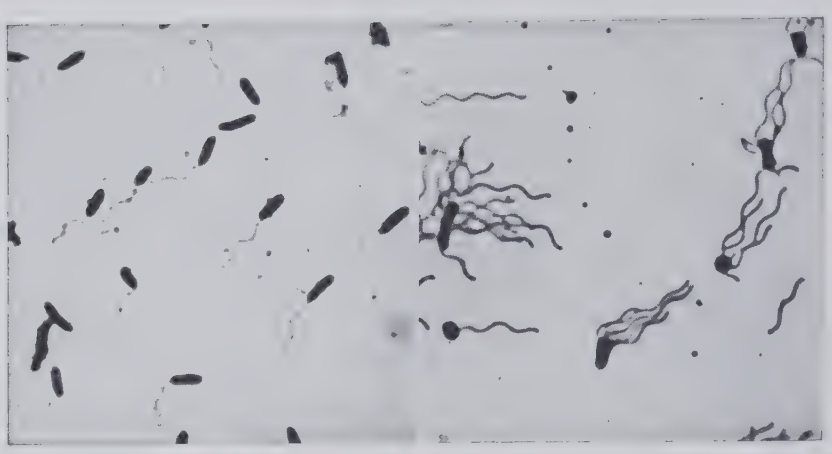


Figure 10. Flagella of bacteria in stained smears examined with the optical microscope. Single polar flagella of a monotrichous bacterium (*Vibrio cholerae*) on the left, and peritrichous flagella (*Salmonella typhimurium*) on the right; × 2000 (Kral).

bacillus, the terms are, for all practical purposes, interchangeable. Among the pathogenic bacteria, the presence of a capsule is associated with virulence, not because of any essential function of the capsule in the economy of the cell, but because it interferes effectively with the ingestion of the bacteria by the phagocytic cells of the body.

Bacterial capsules are not seen in the usual stained smear because of their failure to retain the dye. They may be faintly stained by some procedures,[34] and alcian blue has been reported[81] to stain the capsular polysaccharide, but in general the most satisfactory method of demonstrating the capsule is the "relief" preparation, wet or fixed and dried, in which the bacteria are suspended in diluted India ink (colloidal carbon). The capsule displaces the ink, and the bacterial cells appear to lie in lacunae, representing the capsules, in the dark background.

The relative size of the capsule varies widely. In heavily encapsulated forms, such as pneumococcus and Friedländer's bacillus, the thickness of the capsule is frequently greater than the diameter or breadth of the cell and is often continuous over adjacent cells such as diplococci or streptococci. In other bacteria it may be much smaller, no more than a thin layer about the cell, but still demonstrable, and in still others the capsule is too small to be directly demonstrable even though capsular material may be shown to be present by chemical and immunological methods. The size of the capsule is somewhat dependent upon the method used for its demonstration, *i.e.*, drying and fixing tends to shrink it; it is generally agreed that wet relief preparations give the most accurate results.

The nature of capsular substance. The nature of the material making up the bacterial capsule has been of considerable interest, in large part because in a number of instances the formation of antibody to the capsular substance of pathogens is the most significant element of the effective humoral immune response.

The capsular substance of most, but not all, bacteria is polysaccharide which may or may not contain nitrogen, and which may give sugar acids, amino sugars, and organic acids as well as monosaccharides on hydrolysis. For example, the capsular material of type 2 pneumococcus is a polymer of glucose. That of type 3 pneumococcus is more complex and consists of glucose and glucuronic acid linked at the 1 and 4 positions respectively to give 4-β-glucuronosidoglucose, or cellobiuronic acid, and these aldobionic acid units are polymerized by union at positions 1 and 3 to give chains having molecular weights as high as 140,000. The capsular substance of type 1 pneumococcus contains nitrogen

and appears to be a polymer of trisaccharide made up of two uronic acid (in part galacturonic acid) moieties together with a third component containing nitrogen and acetyl groups.

Some bacterial capsules may contain mucoprotein admixed with polysaccharide, but whether this is to be regarded as a constituent of the capsule or a contaminant is not clear. However this may be, the marked superiority of the encapsulated bacterial cell to purified polysaccharide in stimulating antibody formation suggests that at least some of the polysaccharide may be linked to protein in the living cell.

Other bacterial capsular materials, especially in the genus Bacillus, are polypeptide in nature. That of the anthrax bacillus is a D-glutamyl polypeptide, while those of nonpathogenic species contain both D-glutamic acid and the naturally occurring isomer also, to as much as 20 per cent. In these forms the polypeptide does not appear to be mixed with polysaccharide. The capsular material of the tubercle bacillus seems to be a special case in that it contains relatively large amounts of nucleic acids admixed with polysaccharide.

The capsule is a loose structure that does not interfere with permeability. Since the enzymatic depolymerization of the capsular substances, such as the pneumococcal polysaccharide and the hyaluronic acid capsule of some hemolytic streptococci, appears neither to damage the cell wall nor affect viability, the capsule is regarded as a structure distinct from the cell wall in many bacteria. In some cases, however, notably the glutamyl polypeptide of Bacillus, there is evidence that depolymerization results in some damage to the cell wall, and it may be inferred that the capsular substance is also a part of the cell wall structure.

In a more restricted sense the capsule hardly attains the dignity of a cell structure in that the capsular substance is an end product of metabolism that is continuously secreted, or excreted, into the surrounding environment. Thus its size is a function of the rate of its formation and secretion and of its continuous solution at the periphery. While in most cases it is presumably formed intracellularly, in some instances it may be produced outside the cell, perhaps at the cell surface. For example, cell-free culture fluid of *Leuconostoc mesenteroides*, a bacterium associated with slime formation in the sugar refining processes, polymerizes sucrose to the dextran capsular substance of this bacterium.

The specific capsular reaction. As indicated above, the capsular substance is antigenic, functioning largely as a partial antigen or haptene. It reacts specifically with homologous antibody to produce a microprecipitation in the capsule, altering its refractive index so that it appears better defined in wet unstained preparations.

This phenomenon was described in 1896 by Rogers, working with the fungus Oidium, and by Neufeld in 1902 for the pneumococcus, as a swelling of the capsule, the Quellung reaction. This reaction allows rapid serological identification and has been applied to several groups of streptococci, the meningococcus, the influenza bacillus, Friedländer's bacillus, some of the coliforms, and to Pasteurella and Bacillus species, as well as to the pneumococcus. There appears to be some question as to whether actual swelling occurs. Careful measurement has shown that in most cases an increase in size cannot be demonstrated, but in some instances, such as pneumococcus, it may occur. Since one pneumococcus combines with 4×10^6 antibody molecules which, when packed, have a volume of about 0.8 μ^3, but increases in volume from 2.8 to 9.8 μ^3, swelling in addition to the volume occupied by the immune globulin has occurred.[62] Such swelling may be a consequence of several factors such as a secondary hydration and an increased viscosity that retards diffusion.

The reaction of capsular substance with immune serum globulin also occurs with other proteins under appropriate conditions. It appears to consist of a salt-like combination between the capsular substance and proteins such as casein,

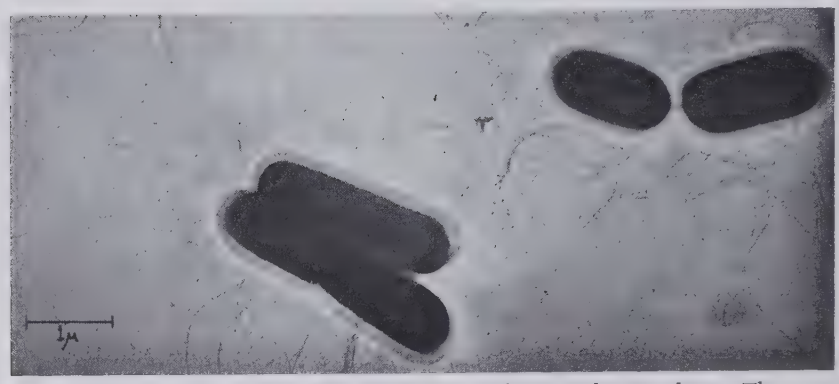


Figure 11. Electron micrograph of *Clostridium tetani* from 24-hour culture. The protoplasm is homogeneous at this stage, and the clear cell walls are apparent. Peritrichous flagella are shown also, and the bacilli are in various stages of cell division. (Mudd and Anderson.)

various albumins and globulins, and hemoglobin at a low pH on the acid side of the isoelectric point of the protein.[137] This capsular reaction differs from that with homologous antibody in that it is nonspecific and pH dependent.

The Cell Wall and Plasma Membrane

The occurrence of bacteria in shapes other than spherical is evidence of a rigidity of structure sufficient to withstand the forces of surface tension. While the gel-like state of the protoplasm may contribute in part to such rigidity, the cell structure largely responsible is the cell wall. It was not possible to demonstrate unequivocally the presence of a cell wall in bacteria until relatively recently when newer techniques, especially electron microscopy, became available. It is now apparent that the outer structure of the bacterial cell is dual in nature and consists of the cell wall proper and the plasma membrane which lines the inner surface of the cell wall.

The Cell Wall.[109, 110, 111, 112, 146] The cell wall is not apparent in the usual stained smear, but when the cytoplasm has been shrunk away by boiling the cells in dilute alkali it is demonstrable in crystal violet-stained smears. It may also be stained by Dyar's method in which Congo red is used with cetyl pyridinium chloride as a cationic mordant.

When the bacterial cell is shattered by physical means such as sonic or ultrasonic vibrations, the cell wall fragments do not tend to round up but show jagged lines of fracture. In electron micrographs of cell wall fragments the structure appears to be a collapsed sac, but this is an artifact due to the effects of surface tension forces operative during the process of drying. When the cell walls are dried from the frozen state this is avoided, and rod-shaped forms, for example, are cylindrical in shape. Data such as these strongly support the concept of the cell wall as a structure having rigidity as well as ductility and elasticity apparent in the intact cell.

Cell wall preparations. Bacterial cell walls may be prepared free of intracellular material in a number of ways. Mechanical disintegration of the cells has the advantage that it avoids chemical alteration of the cell wall substance, provided that autolysis by the freed microbial enzymes does not occur. This is accomplished by grinding, shaking with small glass beads, or by sonic or ultrasonic disruption.

Bacteria vary in their resistance to such mechanical disruption. In the Mickle tissue disintegrator,[85] in which the bacterial suspension is mixed with small glass beads such as glow beads and the containers shaken by a vibrating tuning fork, the time required for disruption of 95 per cent or more of the cells varies from 5 minutes for fragile bacteria such as the cholera vibrio to as much as an hour for staphylococcus.

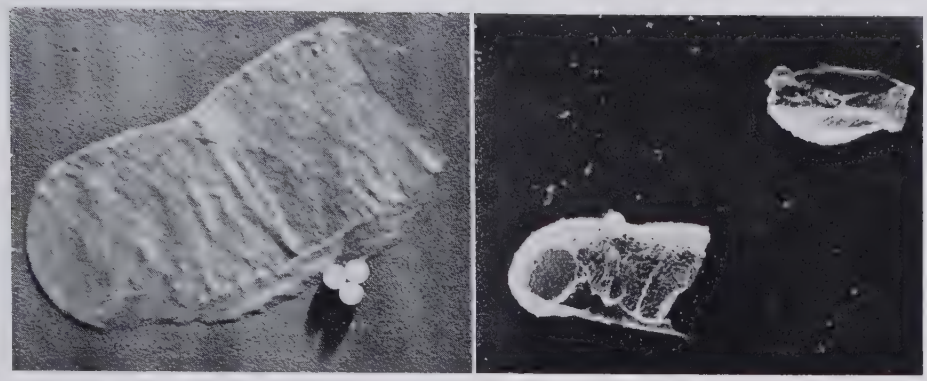


Figure 12. Cell walls of *Bacillus megaterium*. *Left*, an air-dried preparation which superficially exaggerates the size of the cell; the thickened band represents cell wall synthesized during the early stages of cell division, and the spherical particles are latex. × 11,500. *Right*, freeze-dried preparation showing the three dimensional structure of the cell wall, corresponding more closely to the size of the intact cell. × 12,250. (Salton and Williams.[113])

Similar results are obtained by subjecting the bacterial suspension to sonic vibration at 9 to 10 kc. or ultrasonic vibration at 20 to 40 kc. Some bacteria are highly resistant to the latter kind of treatment, especially staphylococci and streptococci, and in general gram-positive bacteria are more difficult to break up by mechanical means than are gram-negative bacteria. The cell walls are separated from the liberated cell contents by centrifugation and, after repeated washing with distilled water or molar sodium chloride solution, are free of electron-dense particles, nucleic acids, etc. Cell walls so prepared retain substantially all their original properties, for example, will adsorb bacteriophage (Chap. 4), are stable and resistant to digestion with trypsin, ribonuclease, and deoxyribonuclease.

Properties of the cell wall. Studies on such preparations have shed considerable light on the nature of the cell wall. It is thinner in gram-negative bacteria than in gram-positive bacteria, ranging in general from 10 to 25 mμ in thickness, makes up about 20 per cent of the dry weight of the cell, has an effective pore diameter of 1 to 2 mμ, and is permeable to molecules as large as nucleotides.

While the cell walls of some kinds of bacteria appear to be homogeneous in electron micrographs, those of others, notably Spirillum, are made up of spherical macromolecules, ranging from 50 to 150 mμ in diameter but constant within species, arranged in regular, hexagonal or rectangular, patterns.[60, 67, 113] Assuming a regular packing of such macromolecules, the interstices correspond approximately to the effective pore diameter determined by permeability studies.

The cell wall substance consists of peptide, polysaccharide, and lipid, highly individualistic in kind and amount in different bacterial species.[147] The peptide fraction is characterized by the presence of relatively large amounts of glutamic acid and alanine. That found in gram-positive bacteria lacks arginine and aromatic and sulfur-containing amino acids which are found in the cell walls of gram-negative bacteria, and the latter appear to contain considerably more, perhaps as much as tenfold, lipid than the former. Pimelic acid is found in the cell walls of some, but not all, bacteria. Relatively large amounts of phosphorus are present as ribitolphosphate and glycerophosphate polymers. These complexes contain other substances, *e.g.*, α-glucosyl and O-alanyl residues, and are called teichoic acids.

The polysaccharides hydrolyze to sugars such as rhamnose, galactose, glucose, mannose, and arabinose. That of the diphtheria bacillus has been found[58] to be an oligosaccharide made up of 2 molecules of D-galactose, 1 of D-mannose, and 3 of D-arabinose, and to have a mo-

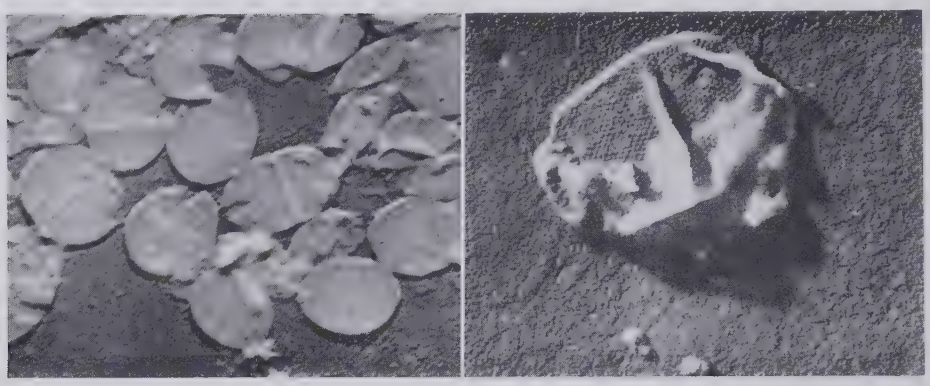


Figure 13. Cell wall preparations. *Left,* unwashed cell walls of *Streptococcus fecalis* prepared in the Mickle apparatus, showing the splitting of the cell wall which allows the contents to escape, the small electron-dense bodies adhering to the cell wall, and the thickened bands occurring in the early stages of cell division. × 12,000. *Right,* freeze-dried preparation of the cell wall of *Rhodospirillum rubrum* showing the spherical bodies of the cell wall of this microorganism. × 42,000. (Salton and Williams[113]; Salton and Horne.)

lecular weight of about 1000. Hexosamine is found regularly and probably exists in the cell wall as N-acetylhexosamine though not as a chitin-type polymer.

While, as indicated above, bacterial cell walls are not digested by trypsin, those of a number of kinds of bacteria, including certain of the micrococci and Bacillus, are broken down by lysozyme, the bacteriolytic agent found in lacrimal and other secretions, egg white, etc. The substrate of lysozyme in the bacterial cell wall is a hexosamine-containing mucocomplex or mucopolysaccharide, but the enzymatic breakdown is incomplete in that the major products of degradation have molecular weights of 10,000 to 20,000. The dissolution of the cell wall by lysozyme, then, is not a matter of complete digestion, but rather a degradation of a component of the cell wall that may bind the major peptide and polysaccharide components, with breakdown of the cell wall as a structure. It has been suggested that lysozyme hydrolyzes the β 1-4 linkages between N-acetylmuramic acid and N-acetylglucosamine, and that the cell walls of lysozyme-sensitive bacteria contain appropriately linked repeating units of these substances.

The cell walls of streptococci are resistant to the action of lysozyme but are broken down by enzymes produced by *Streptomyces albus* and other actinomycetes.[80, 106, 107, 108] Bacterial cell walls attacked by lysozyme are not affected by these enzymes, indicating that a different substrate is involved. The products of degradation are, however, similar to those produced by lysozyme.

The Plasma Membrane. The plasma, or cytoplasmic, membrane* of the bacterial cell lies on the inner surface of the cell wall, separating it from the cell plasma. On mechanical disruption of the cell, fragments of the membrane may be seen attached to the broken cell wall, and it may be stained in the cell by appropriate technique.[103] The membrane, on separation from the cell wall and debris of fragmented cells, breaks up into the so-called small particle fraction that may be removed by differential centrifugation.

The plasma membrane substance appears to be a complex lipoprotein, containing 40 per cent protein and 20 to 25 per cent lipid, and makes up about 10 per cent of the dry weight of the cell.[88] These and related data may be taken to indicate that it conforms to the classic concept of lipoprotein membranes and is, in the intact cell, a closely packed lipoprotein sheet, 2 to 4 molecules thick, and about 15 mμ thick in the hydrated form.

* The term plasma membrane is used in preference to cytoplasmic membrane because it does not imply a precisely defined cytoplasm portion of the bacterial protoplasm.

This membrane has little mechanical strength and does not contribute significantly to maintenance of cell shape as shown by the rounding up of the bacillary form after removal of the cell wall by enzymatic digestion (see below). It is forced against the cell wall by a hydrostatic pressure, as much as 20 atmospheres, corresponding to the osmotic pressure differential across it. While it is a structure distinct from the cell wall in that the two differ in chemical composition and enzyme content, the two are joined by more than lateral cohesion. Thus, the plasma membrane is broken up in the absence of the cell wall by chilling, but much less readily in the intact cell, and it may be inferred that some sort of bonding occurs between the two structures such that rearrangement of parts of the membrane to give freely permeable areas is inhibited.

Osmotic Regulation.[89] While the cell wall and plasma membrane are distinct, artificially separable structures, in the bacterial cell they make up an integrated unit whose function is, in part, that of an osmotic barrier and regulatory mechanism separating the cell plasma from the suspending medium and implementing, for the bacteria, Claude Bernard's concept of the relatively isolated environment required by free-living organisms.

The contribution of the cell wall to this joint function is largely that of a supporting structure, but it also contributes to the permeability of the intact cell in that it acts as a barrier to substances having molecular weights of 10,000 or more. While this is not essential to the physiological economy of the cell (see below), it does provide some protection against large molecular weight substances such as antibodies and lytic enzymes. The plasma membrane functions as the significant osmotic barrier to lower molecular weight substances and exhibits the usual properties of living semipermeable membranes such as selective permeability and the transport of substances across the barrier against concentration gradients.

The bacterial cell is apparently freely permeable to the sodium ion, with rapid equilibration between internal and external concentrations. The potassium ion, however, may accumulate intracellularly against a concentration gradient; the process is associated with exergonic metabolism and is inhibited in the presence of 2-4-dinitrophenol, sodium azide, and similar respiratory poisons.

Relatively precise studies of bacterial permeability to amino acids, lysine and glutamic acid particularly, have been carried out with a few species of bacteria, and there appear to be significant differences between the gram-negative and gram-positive forms. It is generally said that the gram-negative bacteria are freely permeable to amino acids while the gram-positive bacteria are not. This appears not to be strictly true for, on the one hand, the occurrence of internal amino acid pools is demonstrable in at least some gram-negative bacteria, and, on the other, the gram-positive bacteria studied seem to be at least partially permeable to some amino acids although the accumulation of others involves active transport and a source of energy. Thus, while lysine appears to diffuse readily in both directions across the osmotic barrier, and is independent of exergonic metabolic activity, at the same time intracellular lysine cannot be washed out of bacterial cells suspended in distilled water unless glucose is present.

Permeases.[29] There is evidence that the active transport of nutrient substances across the osmotic barrier is a function, at least in part, of enzymatic activities to which the name permeases has been given. Such a permease is essential to the utilization of lactose by some strains of coliform bacilli, *e.g.*, the appropriate enzyme, β-galactosidase, may be present within the cell, but unless the transport mechanism is present, the sugar is not utilized. The permease activity is genetically controlled, and cells which lack it, but contain β-galactosidase, are designated cryptic mutants (Chap. 7), *i.e.*, are metabolically inert in the presence of the substrate. A considerable number of such permease activities have been described.

Bacterial permeability is of particular interest in connection with the nature of the effect of antibacterial substances characterized by bacteriostatic or growth-inhibiting activity such as the chemotherapeutic drugs, the basis of reversal of antibacterial activity, and the nature of drug resistance as related, for example, to altered permeability to amino acids such as glutamic acid.[40] The evidence, though meager in contrast with that available for classic subjects of permeability studies such as Arbacia eggs and erythrocytes, suggests that an active transport mechanism[100] or exchange diffusion is involved, with reversible deformation of parts of the plasma membrane to allow passage of the carrier.[86] This raises the interesting possibility that some kinds of antibacterial activity may result from effects on a carrier mechanism and/or the structure of the osmotic barrier.

Bacterial Protoplasts.[21, 83, 141] On enzymatic degradation of the cell wall of the living bacterial cell, as for example by treatment of Bacillus species with lysozyme, the cell first swells, then the protoplasm becomes partially detached from the disintegrating cell wall, and finally, following complete dissolution of the cell wall, the cell content assumes a spherical shape. This spherical form, the cell protoplasm enclosed in the plasma membrane, is the protoplast.

The protoplast is a fragile structure because the plasma membrane has little mechanical strength. When the cell wall is removed by enzymatic dissolution in physiological saline, the protoplasts begin to break up almost as soon as they are formed, first to an empty membrane or "ghost," followed by fragmentation of the membrane.[138] It was found by Weibull that sucrose and polyethylene glycol act as stabilizing agents, and it is now established that if the cell wall is removed from cells suspended in mediums such as 0.1 to 0.5 molar sucrose, 7.5 per cent polyethylene glycol, 0.25 to 0.5 molar sodium chloride, or 0.5 molar phosphate at pH 7, the protoplast is relatively stable over a period of several hours if kept under semi-anaerobic conditions and protected from shock. Stabilized protoplasts suspended in 0.2 molar sucrose solution containing magnesium salts at concentrations of 0.01 molar or greater may be lysed by reduction of the sucrose concentration to give stable "ghosts." When the cell wall is damaged so as to affect its structural integrity, but remains present, the bacterial cell also rounds up in spherical form, and is known as a spheroplast.

Physiological studies on stabilized protoplasts have shown that the competence of the intact cell persists almost, but not quite, entirely in the protoplast and indicates the function of the cell wall by implication. Protoplasts show a high endogenous respiratory rate ($QO_2 = 66$) and can oxidize glucose. They are able to incorporate C^{14} in protein and

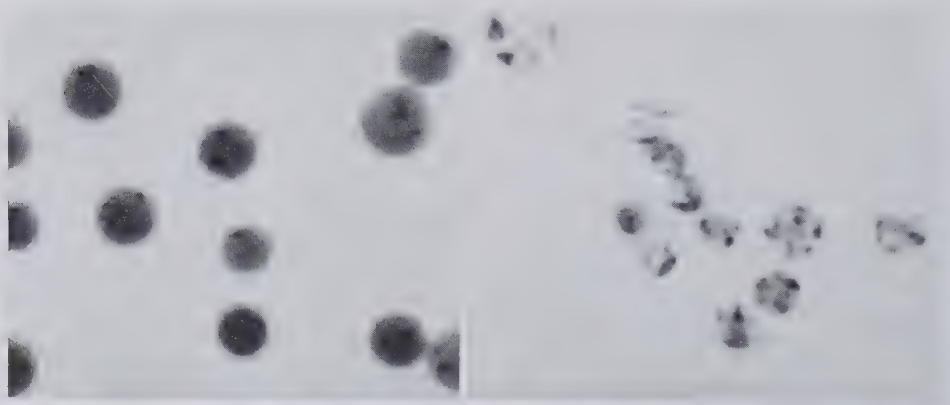


Figure 14. Protoplasts (*left*) and protoplast ghosts (*right*) of *Bacillus megaterium*. Note the spherical shape assumed by the bacillary form on dissolution of the cell wall. Phase contrast photomicrographs. × 3000. (Weibull.[140])

nucleic acid at rates only slightly less than those of actively growing intact cells. The path of incorporation of acetate carbon is consistent with the operation of the tricarboxylic acid cycle in providing intermediates for amino acid synthesis[82] indicating that actual synthesis rather than exchange is involved, and the synthetic abilities of the protoplast are further substantiated by the synthesis of the adaptive enzyme β-galactosidase. There is some evidence also that the protoplast is capable of growth and division.

The protoplast differs from the intact cell in that it can form spores (see below) and support bacterial virus multiplication (Chap. 4) only after these processes have been induced in the intact cell. It may, however, be infected with bacteriophage infectious nucleic acid, suggesting that the cell wall is functional in the initial adsorption and discharge of the viral DNA into the host cell, but does not affect subsequent synthesis. Thus, spores are formed by *Bacillus megaterium* protoplasts only when the process of sporogenesis has been initiated prior to dissolution of the cell wall by treatment with lysozyme, but the nature of the contribution of the cell wall or consequence of its presence to the initiation of sporogenesis is not known. Similarly, the first stage in the infection of the host bacterium with bacteriophage is a specific adsorption of the phage particle to the cell wall, and phage is not adsorbed by the protoplast. If, however, the protoplast is formed after the initial stage of infection has occurred, the virus multiples in the protoplast.

Internal Structures[72,92]

The dividing line between external and internal structures of the bacterial cell is not a sharp one and is perhaps justified for the most part on little more than a utilitarian basis. Thus, in a sense the protoplast is a subcellular element, yet flagella remain attached to it since the basal granules at or near the inner surface of the cell wall in which they originate are apparently not an integral part of the cell wall structure because they remain after enzymatic dissolution of the cell wall. Similarly, the bacterial spore is formed within the cell, but it represents a stage in the life history of the bacterium rather than a subcellular structure in the ordinarily accepted sense.

The Bacterial Spore.[39, 49, 102, 118, 132] Spore formation is uncommon among the bacteria and is confined largely, if not entirely, to the bacilli. The aerobic spore-forming bacilli make up the genus Bacillus, and the obligate anaerobic or microaerophilic bacilli that form spores are members of the genus Clostridium. Both are widely distributed in nature. Members of the former group are saprophytic forms found in soil, dust, and water and, with the exception of the anthrax bacillus, are not pathogenic for man under ordinary circumstances. The anaerobic group is similarly distributed and includes both saprophytic nonpathogenic forms together with microorganisms which are pathogenic by virtue of toxin formation, such as the tetanus and gaseous gangrene bacilli and the botulinus bacilli, although they are not obligate parasites.

The bacterial spore is a refractile oval body formed within the bacterial cell and found both intracellularly and extracellularly in the usual stained smear; the vegetative cell substance has disintegrated about those lying free. Within the cell, the long dimension of the spore is parallel with the long axis of the bacillus. Its breadth may be essentially the same as the width of the vegetative cell, or it may be greater and bulge the vegetative cell wall. The latter appearance is most commonly found in the anaerobic forms and is the basis of the generic name Clostridium.

The spore may be located in the center of the vegetative cell, where it is said to be *central,* or it may be found part way between the center and the end of the cell and be *subterminal,* and finally it may occur at the end of the cell and is designated *terminal.* The relative size

Figure 15. Spore formation by bacteria. *Left,* the anthrax bacillus, which forms central spores which do not distend the wall of the vegetative cell; the tendency of the bacilli to occur in chains is also characteristic. *Center, Clostridium botulinum* type B, which shows the typical clostrispores separated from disintegrated vegetative cells. *Right, Clostridium sporogenes,* showing a subterminal clostridial spore. These preparations are all stained with a single stain in the usual way; the vegetative cells take up the dye, but the spores remain unstained. Fuchsin; × 1050.

of the spore is constant, and its location reasonably so within species. Its size and location may result in a characteristic morphology, *e.g.*, the large terminal spore of the tetanus bacillus gives the spore-containing vegetative cell a drumstick appearance.

In the usual stained smear the spore appears as an unstained refractile body within the stained vegetative cell, and some stain may adhere superficially to free spores to accentuate their outlines. The spore may be stained by using heat and/or mordants. Once stained, it is equally difficult to decolorize with the usual reagents such as alcohol, acid-alcohol, and alcohol-acetone. Consequently, differential staining of the spore is a simple matter; for example, the smear may be stained with hot carbolfuchsin, decolorized with alcohol, and counterstained with a dye of contrasting color, such as methylene blue to give a red spore in a blue vegetative cell.

The factors involved in spore formation are poorly understood, but in general spores are formed most readily under optimal conditions for growth, and spore formation begins toward the end of, or just following, the period of exponential growth. There is no reason to believe, as is sometimes supposed, that spore formation occurs in response to adverse environmental conditions.

The effects of environmental conditions on spore formation vary from one kind of bacteria to another. For example, the aerobic anthrax bacillus forms spores only under aerobic conditions, and spores are not found in impression smears of organs such as spleen from animals dead of anthrax since the tissues are not sufficiently aerobic to permit sporulation. Similarly, the anaerobic sporulating bacilli do not form spores under aerobic conditions that do not allow growth. The temperature of incubation may be a factor also, and it is common knowledge that aerobic sporulating bacilli do not form spores when grown at maximal temperatures tolerated for growth, and, in fact, permanently asporogenous variants may be produced by continued cultivation at high temperatures.

Cytological observations have shown that at the initiation of spore formation there is an assembling or local concentration of material staining deeply with basic dyes, presumably nucleoprotein, which constitutes the spore primordium or forespore. This may occur with the formation of a granule which enlarges to form the spore in some bacteria, in others there seems to be an aggregation of deeply staining granules, and in still

others only a local concentration unrelated to granules. In any case, when formation is complete the spore assumes the characteristic refractile appearance, and the surrounding vegetative cell sloughs off. Only one spore is formed by a single vegetative cell, and spore formation cannot be regarded as a method of multiplication, and, further, there is no evidence of sexual phenomena or conjugation in the formation of bacterial spores such as occurs among the fungi.

The spore does not differ materially in chemical composition from the vegetative cell other than a somewhat higher content of calcium and nitrogen. It has been found,[33] however, that the spore contains antigens that are not present in the vegetative cell, and this indicates that the process of sporulation is more than an assembling and concentration of vegetative cell substance. The spore antigens are specific in that spores from immunologically different vegetative cell species are distinct and do not share common spore antigens. The spore contains a full complement of the enzymes present in the vegetative cell, though in reduced amount, and is not dormant in that the low endogenous respiration of moist spores is measurable in the usual respirometers.

The composition of the spore does not account satisfactorily for its unusual resistance to injury. It is not only relatively impermeable to dyes, as noted above, but also is much more resistant to the injurious effects of heat, drying, many bactericidal chemical compounds, etc., than is the vegetative cell.

Sterilization procedures (Chap. 6) are necessarily directed toward the destruction of the ubiquitous microbial spore. Some bacterial spores, such as those of organisms causing flat sours in canned foods, may be extremely resistant to heat and require heating in steam under pressure at 120° C. for as long as 3 hours to insure destruction. Most are not this resistant and are killed by moist heat at 115 to 120° C. for 15 to 20 minutes. Still others are destroyed by boiling for a short time, but few if any are killed by treatment at 58 to 60° C. for 30 minutes which suffices to destroy most bacterial vegetative cells. They are also extremely resistant to drying; the classic example is that of the viability of anthrax spores dried on silk threads in Koch's laboratory after 60 years.

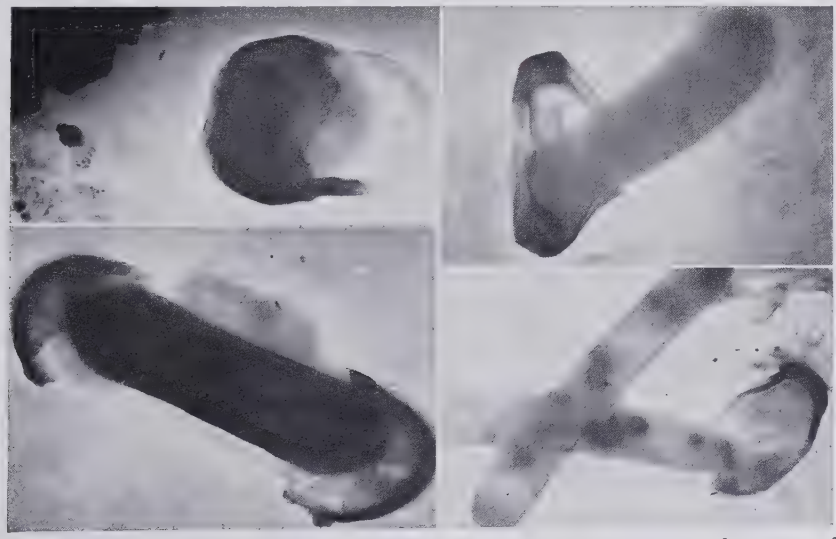


Figure 16. Electron micrographs illustrating the process of germination of spores of *Bacillus mycoides*. In the upper left the vegetative cell is just beginning to emerge from the spore case. In the upper right the vegetative cell is growing out of the side of the spore case and has broken the case in two parts in the lower left. In the lower right germination is complete. The darker areas in the vegetative cells are regarded by some as nucleus-like in nature. (Courtesy Dr. G. Knaysi and the Journal of Bacteriology.)

The ratio of the concentration of bactericidal compounds lethal to the vegetative cell to that lethal for the spore is on the order of 10^3 to 10^4 for many disinfectants such as hypochlorites and phenols. Interesting and significant exceptions are the alkylating agents such as ethylene oxide or formaldehyde, for which the ratio ranges from 0.5 to 15.

When the spore is placed in an environment favorable to the growth of vegetative cells it germinates.[50] The factors affecting germination are not well known. It may be delayed and irregular, and in some instances this has been found to be associated with the presence of fatty acids, such as oleic and linoleic acids, in the culture medium. In others, germination may be accelerated by heat shock, a short exposure to a high temperature, say 80° C., prior to culture. Once germination is initiated, it occurs at an exponential rate. Nucleic acid metabolism differs from that of the vegetative cell, at least in the case of *Bacillus subtilis,* with a preliminary synthesis of RNA during the first two hours, followed by initiation of DNA synthesis.[145]

The first microscopic evidence of germination is a change in the refractile appearance of the spore, and it becomes more permeable to dyes and takes up dye like the vegetative cell. The sequence of events thereafter is variable. The spore wall may become thin and stretch and assume the shape of the vegetative cell. In other instances the vegetative cell appears to grow within the spore wall which is split and cast off as a hull. The anthrax bacillus grows out of one side of the spore wall, while the closely related *Bacillus subtilis* grows out of opposite sides simultaneously. In *Bacillus megaterium* the spore case splits along the equatorial ridge or groove, allowing the vegetative cell to emerge.[71] Other bacteria have intermediate methods of germination, and irregularities may occur in the development of spores into vegetative cells in the same bacterial species. It is to be noted that, as the vegetative cell produces only one spore, on germination the spore gives rise to only a single vegetative cell, and the process of germination is not one of multiplication.

The biological function of the bacterial spore is not known. It is clearly a normal stage in the life history of the spore-forming bacteria, and may be regarded as a resting stage, possibly analogous to hibernation or estivation in higher forms. Its high resistance to injury serves to carry the bacterial species through unfavorable circumstances, and the spore might be supposed to have survival value.

Staining Reactions. As implied be-

Figure 17. Recently germinated vegetative cells of *Bacillus mesentericus,* showing discarded spore cases. Note the cell boundaries in the long filaments made up of cells attached end to end. The deeply staining areas are chromatinic bodies. Osmium tetraoxide–hydrochloric acid fixation, Giemsa; × 4000. (Robinow.)

Differences Between Gram-Positive and Gram-Negative Bacteria

GRAM-POSITIVE BACTERIA		GRAM-NEGATIVE BACTERIA
more susceptible	antibacterial activity of basic dyes, anionic and cationic detergents, phenol, sulfonamides, penicillin	more resistant
more resistant	antibacterial activity of azides, tellurites, oxidizing agents, streptomycin	more susceptible
more resistant	digestion by proteolytic enzymes, lytic action of alkali	more susceptible
more complex	nutritive requirements	simpler
11–22% lipid	cell wall composition	1–4% lipid
all amino acids present		lacking some amino acids

fore, the intact bacterial cell stains readily with basic dyes such as crystal violet, methylene blue, and basic fuchsin but relatively poorly with acidic dyes such as eosin. While irregular staining with differentiation of more deeply staining areas or granules is observed in old cells and is characteristic of a few kinds of bacteria, most bacteria stain uniformly without the differentiation of internal structure that is readily demonstrable in, for example, mammalian tissue cells.

This marked affinity for basis dyes is indicative of an acidic protoplasm containing unusually large amounts of nucleoprotein more or less uniformly distributed. This inference is substantiated by a high content of purine and pyrimidine nitrogen in the bacterial cell, and ribonucleic and deoxyribonucleic acids make up from 5 to as much as 30 per cent of the dry weight of the cell substance.

The staining reactions of the intact bacterial cell are remarkably uniform with two important exceptions. These are the reaction to the Gram staining procedure and the high resistance to both penetration of dye and decolorization characterizing the so-called acid-fast bacilli.

The Gram stain.[12] This differential staining procedure was devised by the histologist Christian Gram as a method of staining bacteria in tissues, and described in 1884. It is an arbitrary procedure consisting of four steps, *viz.*, (1) primary staining with crystal violet, usually containing a mordant such as ammonium oxalate; (2) the application of dilute (1:15) Lugol's iodine solution; (3) decolorization, most commonly with 95 per cent ethanol; and (4) counterstaining with a dye of contrasting color, usually safranin. When bacteria are stained by this method, they are separated into two groups. The gram-positive bacteria are those which retain the primary stain and are deep violet in color, and the gram-negative bacteria are those which are decolorized and are lightly stained by the counterstain, pink in the case of safranin.

The gram-positive reaction is relatively rare. It occurs only among the bacteria, yeasts, and filamentous fungi. A very few biological structures are gram-positive; they are those which are thought to be autoreproducing, and they include chromosomes of certain species, mitochondria, centrosomes, and centromeres.

The reaction of bacteria to the Gram stain is correlated with a number of other characteristics, and representative differences are shown in the accompanying table. Such differences are only relative and are not completely consistent. The gonococcus, for instance, though gram-negative, behaves in many respects, such as susceptibility to the antibacterial activity of penicillin, as if it were gram-positive.

There are also significant differences in the permeability of bacterial cells to

amino acids, glutamic acid and lysine in particular, that are associated with the Gram reaction. Gram-positive bacteria are distinguished by their ability to concentrate such amino acids from the environment, while their synthesis occurs intracellularly in gram-negative bacteria,[41] an observation that may be linked with the relative complexity of the nutritive requirements of the gram-positive forms. Differences between bacteria correlated with the Gram reaction reach an impressive total and suggest that reaction to the Gram stain is a reflection of fundamental differences between the two kinds of bacteria. Consequently, the mechanism of the Gram reaction has been of considerable interest.

Three kinds of theories have been proposed to account for the Gram stain reaction. A differential permeability to the alcohol-soluble dye-iodine complex was proposed by Benians; a difference in iso-electric point of the cell contents between the gram-positive and gram-negative bacteria was urged by Stearn and Stearn on the assumption that a lower iso-electric point in the gram-positive bacteria would result in a more stable complex with the basic dye of the primary stain; and, finally, it has been postulated, largely by Churchman, that the site of the Gram reaction is in the outer layers, or cortex, of the cell, the internal material being gram-negative in both kinds of bacteria.

Each of these is supported to some degree by available evidence, but none to the exclusion of the others. In general, the Gram reaction appears to be a property of the entire cell and to be associated with some cell components. The most precise evidence in this connection is the presence of a magnesium ribonucleate–protein complex in the gram-positive bacteria which is not present in gram-negative bacteria. This material can be removed from gram-positive cells by solution in bile to leave a gram-negative "cytoskeleton" and redeposited to make the cells gram-positive again. Gram-positive bacteria may also be made gram-negative by treatment with ribonuclease, and it has been possible to replace the bacterial ribonucleate with yeast ribonucleate. It has not, however, been possible to make naturally occurring gram-negative bacteria gram-positive by treating with the ribonucleate complex obtained from gram-positive bacteria.

There is also evidence associating the Gram reaction with other cell components, lipid and polysaccharide. Nevertheless, the mechanisms operative in the Gram reaction are still poorly understood, and the basic differences between the two kinds of bacteria, associated with or reflected in other properties, are not resolved.

The acid-fast stain. It was early observed that some kinds of bacteria are difficult to stain and equally resistant to decolorization with highly effective agents such as acid-alcohol, and because of the latter were termed acid-fast. In this respect they resemble superficially the bacterial spore. These bacteria form a homogeneous group, the acid-fast bacteria, making up the genus Mycobacterium, which includes the tubercle and leprosy bacilli together with pathogens of cold-blooded animals and saprophytic forms.

These bacteria are characterized by a high, as much as 40 per cent of the dry weight, lipid content. These bacterial lipids have been studied in some detail, especially those of the tubercle bacillus, and have been found to consist largely of phospholipid and wax. The unsaponifiable portion of the wax fraction includes higher alcohols, one of which, a saturated hydroxymethoxy acid named mycolic acid, is associated with the property of acid-fastness. Mycolic acid may be regarded as the substrate in this staining reaction, and the property of acid-fastness, confined to these bacteria and some actinomycetes, appears to be a consequence of a relatively greater solubility of the phenol-dye in the cellular lipids than in the decolorizing agent.

Neither the Gram reaction nor the acid-fast property of some bacteria gives information about the morphological structure of the bacterial cell, but they are concerned with its chemical struc-

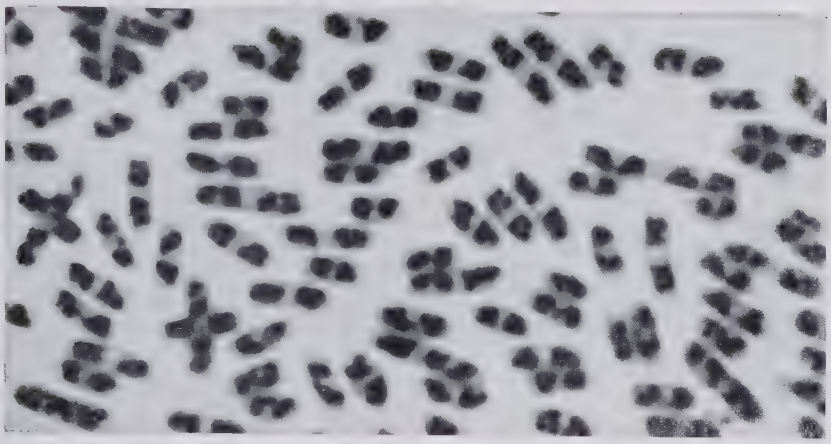


Figure 18. Light microscope photomicrograph of a 2½-hour culture of colon bacilli, fixed with osmium tetraoxide vapor and alcoholic mercuric chloride, hydrolyzed at 56° to 60° C. with normal hydrochloric acid, and stained with Giemsa. The deeply staining bodies are chromatinic bodies. (Robinow.)

ture. In the first instance chemical structure is associated with other properties of the cell as noted above, and in the second with immunological phenomena and pathogenesis in the case of pathogenic forms.

The Bacterial Nucleus. The uniform staining of the bacterial cell with basic dyes having an affinity for acidic, nucleic acid–containing components of protoplasm, without localization of deeply staining material as a morphologically distinct structure, has led to considerable uncertainty as to the existence of a nucleus in the bacteria. At the same time, the functioning of a heredity-controlling mechanism is obvious from the persisting identity of bacteria from one generation to the next.

It has long been known that nucleoprotein is not only present in the bacterial cell, but present in quite large amounts, and this leads to the inference that the chemical equivalent of a nucleus is present. Further, studies[74] utilizing microspectrography in the ultraviolet region have shown that bacteria have two nucleotide systems, a deoxyribose type and a ribose type, intimately related to growth and making up a functional but not morphological unit. The deoxyribose type is regarded as the equivalent of euchromatin, while the ribose system corresponds to heterochromatin, and nucleotide formation in the growing culture is inversely proportional to the generation time during the period of exponential growth.

Cytological studies on bacteria in this connection have made use of the Feulgen reaction* in an effort to localize concentrations of deoxyribonucleic acid in the cell. Bacteria give positive Feulgen reactions, with the color sometimes uniformly distributed over the cell, and at other times deeply staining intracellular granules are demonstrable which are regarded as representing local concentrations of DNA. The uniform coloration is due to the plasma membrane which gives a positive reaction. The granular aggregations of Feulgen-positive material are determined in amount and distribution largely by the methods of fixation used[2] but are so consistently observed that it is unlikely that their particulate nature and morphology are wholly artifacts. Of these aggregates, the largest are the chromatinic or chromatin bodies.

The chromatinic body.[101] The marked affinity of the bacterial cell for the usual basic dyes is due for the most part to the presence of relatively large amounts of ribonucleic acid. When this is selectively removed by acid hydrolysis or

* The Feulgen reaction is a microchemical test for the presence of DNA. The aldehyde groups of acid-hydrolyzed DNA react with Schiff's reagent to restore color to the reduced fuchsin, and aggregates of DNA appear stained a violet color. It is specific not for DNA but for free aldehyde.

treatment with ribonuclease, or is deficient as in starved cells growing on minimal mediums, so that it does not mask the DNA, the bulk of the DNA is found to be aggregated, and these aggregates are called chromatinic bodies. They have only a weak affinity for the usual bacterial stains but may be stained with Giemsa and are Feulgen-positive. The usual method of demonstration is hydrolysis in normal hydrochloric acid at 60° C., followed by fixation with osmium tetraoxide and staining with Giemsa.

These bodies are to some degree artifacts in that, for example, they stain much more deeply following removal of RNA by acid hydrolysis than when the RNA is removed with ribonuclease. That they are not artifacts, however, is indicated by the fact that they are demonstrable as lighter areas in the living cell when observed by phase microscopy and in electron micrographs when the cells are not subjected to treatment other than the necessary drying.

The chromatinic body divides when the cell divides, though not necessarily simultaneously with cell division, is plastic in that it is readily deformed from a symmetrical shape, and is continuously homogeneous in that the structure shows no obvious changes in texture before, during, or after cell division. Various attempts to demonstrate an internal structure, such as electron micrography of thin sections of bacteria, have given equivocal results. It has been calculated[105] that DNA fibers are between 0.6 and 1.4 μ in length; since the chromatinic bodies of, for example, the colon bacillus are smaller than 0.6 μ, such fibers, if present, would need to be folded *in vivo*, and patterns in folding might be anticipated.[73]

The possibility that the chromatinic body is the functional nuclear apparatus of the bacterial cell has been an intriguing one but as yet lacks definite evidence to support it. Mitotic figures have been described[32] as occurring in these bodies, but their existence has been questioned,[17, 101] and it has been urged that what have been taken to be mitotic figures are no more than deformed chromatinic bodies whose arrangement in a sequence that would be consistent with an orderly evolution of mitotic figures cannot be construed as evidence for the actual occurrence of such a sequence of events. There is no doubt that the chromatinic body is made up of nuclear material (DNA), though it is not the only intracellular particulate aggregate of this substance in the bacterial cell, but the question of whether it is a nuclear organization is not yet resolved.

Nuclear function. While it is obvi-

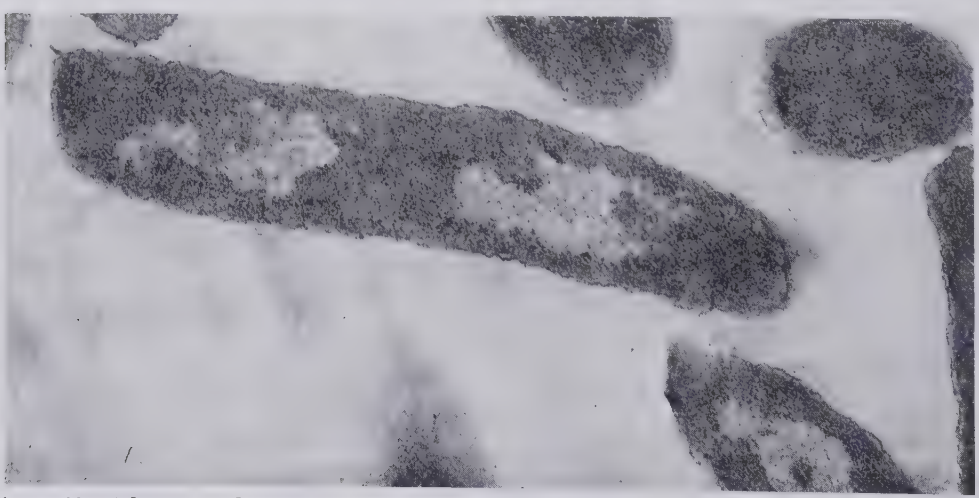


Figure 19. The DNA chromatinic bodies in *Escherichia coli* K-12S during the exponential growth phase. Note the apparent lack of a limiting membrane. Section, fixed in osmium tetraoxide. × 40,000. (Kellenberger.)

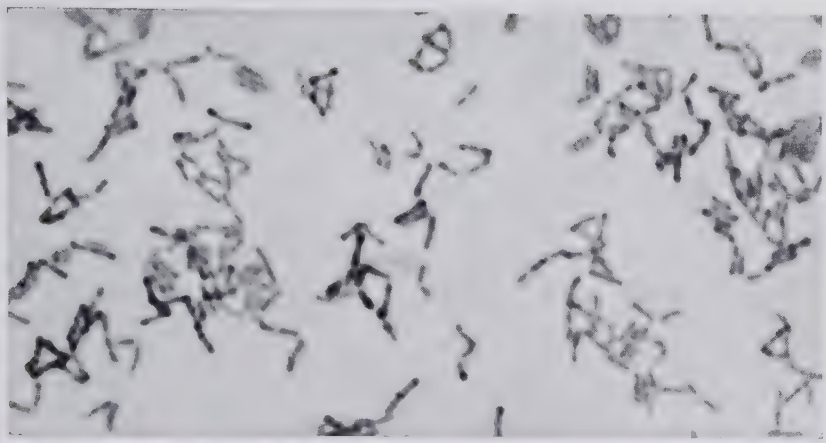


Figure 20. Metrachromatic granules and bipolar staining in the diphtheria bacillus. Note the differences between these organisms and the plague bacilli. Methylene blue; × 1975.

ous that heredity-controlling mechanisms are operative in the bacteria that are not functionally different from those of larger cells, the bacterial cell may not contain structures comparable to the morphological chromosome and chromosome nucleus. The recombination of genetic markers demonstrable in bacteria (Chap. 7) is, of course, solid evidence for the existence of genetic chromosomes in these microorganisms, but a genetic chromosome is a linkage of genic characters that need not have a morphological, in the sense of visible, equivalent.

The significance of size is apparent here for, as pointed out earlier, the physiological processes of bacteria are to be equated against those of larger forms on a molecular, or macromolecular, basis to which morphological structure is secondary. Further, the effective directive, and thus genic, function of DNA in bacterial virus reproduction (Chap. 4) and the phenomena of transduction and transformation in bacterial variation (Chap. 7) suggest that the directive elements in bacterial physiological processes are most profitably considered on a molecular basis. It is unnecessary, and undesirable as well, to postulate that functional structures of larger cells must be replicated in miniature in minute cells such as bacteria.

Protoplasmic Granules.[19] The bacterial cell contains a variety of granules sufficiently large to be seen with the light microscope. Those staining with certain basic dyes are known as metachromatic or Babes-Ernst granules, and are readily observed in bacteria such as the diphtheria and related bacilli stained with alkaline methylene blue or toluidine blue and as polar granules in other forms such as plague bacilli fixed in methanol prior to staining. They are variable in size, to as large as 0.6 μ, and larger granules may represent aggregates of smaller ones. The substance of the metachromatic granules is volutin and is predominantly nucleoprotein in nature. In addition, these granules contain relatively large amounts of trichloracetic acid-insoluble polymetaphosphate whose presence is also associated with their metachromatic character. These granules are dissolved in dilute acid or alkali or boiling water and are characteristically electron-dense but are destroyed in an intense electron beam.

Other visible granules are polysaccharide or lipid in nature. The former stain with iodine to a red-brown color, and the material composing them has been variously termed iogen, granulose, or bacterial starch. Intracellular polysaccharide may also be stained by the periodic acid–Schiff method. Lipid granules have the appearance of vacuoles in bacteria stained with basic dyes but may

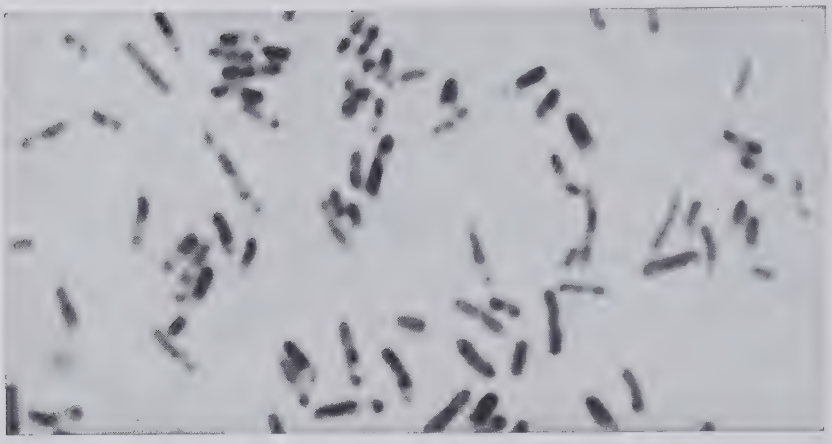


Figure 21. Irregular and bipolar staining of the plague bacillus. Note the heavily stained areas and metrachromatic granules. Fixed in methyl alcohol and stained with methylene blue; × 2400.

be stained with lipid-staining dyes such as Sudan III in wet preparations or Sudan Black in dried smears. In *Bacillus megaterium* the granular lipid has been found to be a polymer of β-hydroxybutyric acid.[140]

The granular polysaccharide and lipid may be regarded as end products of metabolism, and they appear to play no active part in the physiological processes of the cell. Metachromatic granules, however, are the site of enzymatic activity (see below), and there is reason to believe that the contained polyphosphate may serve to store energy conserved in high energy phosphate bonds, accumulated during oxidative processes with adenosine triphosphate (ATP) acting as the intermediate, and released by way of a nucleotide mechanism. The term "reserve substance," which has long been applied to metachromatic granules without supporting evidence, acquires significance in this way.

Submicroscopic particulates. In addition to the granules that may be seen with the light microscope, bacterial protoplasm, like that of other cells, contains minute granules or particulates that are separable from soluble constituents of the cell by centrifugation at speeds giving 100,000 x G or more and may be observed only in electron micrographs.

The sedimentation patterns obtained with bacteria disrupted by various means, and exclusive of larger fragments such as cell wall, metachromatic granules, and chromatophores, consistently show several distinct macromolecular components. There is usually a broad, slowly moving band at 5 S,* and peaks at 8 S, 20 to 30 S, and 40 S.

The 5 S fraction is a heterogeneous mixture of substances making up the ground material of the protoplasm. The 8 S fraction contains the major portion, 80 to 90 per cent, of the DNA present in the cell. The latter suggests that morphological organization of DNA in the intact cells, as for example in chromatinic bodies or other structures that might be thought to have nuclear organization, is fragile inasmuch as constituent DNA is readily liberated in soluble form. Together these two fractions constitute the supernatant and contain half or more of the total intracellular substance measured as nitrogen, protein, or dry weight.

The 40 S fraction makes up the bulk of the sedimentable, particulate material and has been found, on examination by electron microscopy, to consist of spheres 10 to 20 mμ in diameter. It contains the major part of the sedimentable protein and practically all of the cellular RNA. These RNA-rich granules have been of particular interest in connection with the enzymatic activity associated with

* Svedberg units, *i.e.*, 1 S = $10a^{-13}$ cm./sec./unit field.

them. Such particulates are known as ribosomes and probably play a significant part in protein synthesis.[18]

Intracellular Localization of Enzymes[4, 77, 78, 87]

It can be anticipated that the morphological differentiation of the bacterial cell into subcellular structures, both at microscopic and submicroscopic levels, may be associated with a corresponding physiological differentiation and biochemical organization. In fact the simultaneous occurrence of opposing processes, such as the synthesis, utilization, and destruction of nucleotides, within the cell makes some effective separation of metabolic processes inevitable.

The external cell structures, flagella and capsules, have no biochemical function, and their loss does not affect the physiological processes of the cell. The flagella serve only as macromolecular muscles so far as is known, and the capsular substance is a product of metabolism shed into the immediate environment, functional only insofar as it provides protection to the cell from, for example, phagocytosis.

Similarly, the cell wall seems to be largely a supporting structure that contributes in a minor way to the osmotic barrier, but which also may be dispensed with in that the protoplast is physiologically complete with certain exceptions such as its inability to initiate sporulation. It has been reported[42, 43, 44] that cell wall fragments of staphylococcus can incorporate glutamic acid into protein in the presence of ATP and hexosediphosphate, and synthesize catalase and β-galactosidase, but the material consisted of electron-dense spherical bodies enclosed in cell wall fragments. Cell walls washed free of protoplasmic contaminants, as judged by electron micrographs, have not been shown to have enzymatic activity.

The plasma membrane, as the most important element in the osmotic barrier of the bacterial cell, is closely associated with enzymatic activity. The intact membrane gives a positive Feulgen reaction as noted above, and the cytochrome system occurs in close proximity to it.

As noted elsewhere, the relative impermeability of the living bacterial cell to substances such as phosphate, coupled with the vigorous metabolic activity of these microorganisms, implies a highly effective transport mechanism across the osmotic barrier. If, as has been suggested, such a mechanism involved the adsorption of nutrient substances and desorption of end products of metabolism by the appropriate enzymes functioning as carriers, the association of enzymatic activity with the membrane would be very close even though the membrane itself may not have activity. Thus, it is plausible to assume that reduction of the cytochrome–cytochrome oxidase system occurs, at least in part, at the periphery of the cell organization. Further, the facility with which electron-dense particles are trapped in fragments of cell walls of disrupted bacteria suggests that some of these particulates may lie in close proximity to the periphery of the cell.

Further evidence for the localization of enzymatic activity at the cell periphery is provided by, for example, the diffusion of glutamic acid into the cell and the association of glycolytic mechanisms with the cell surface. Glutamine penetrates the cell rapidly while glutamic acid does not unless a source of energy such as glucose is available, implying that the mechanisms of assimilation against a concentration gradient may involve decarboxylation at the cell surface. Similarly, the phosphorylation of hexose by yeast cells appears to occur largely at the periphery of the cell, and the enzymes catalyzing the dephosphorylation of adenosine diphosphate and inorganic pyrophosphate are localized there.

There appears to be little doubt that the microscopic polymetaphosphate-containing metachromatic granules serve as a source of energy in high-energy phosphate bonds, but the way in which the energy available in these discrete "power houses" is tapped for endothermic meta-

bolic reactions is unknown other than that it is released by way of ATP. There is reason to believe that these microscopic (0.2 to 0.4 μ) granules may also function in a manner analogous to that of the mitochondria of animal cells in that cytological evidence suggests that they may be loci of oxidation-reduction reactions.

Treatment with tetrazoles, such as triphenyltetrazolium chloride, results in reduction to insoluble formazan which is seen to accumulate in localized areas. Similarly, indophenol blue appears in localized areas following treatment of the cell with the Nadi reagents, α-naphthol and dimethyl-*p*-phenylenediamine. It has been objected that formazan accumulates by accretion in any case, and that indophenol blue may accumulate by solution in lipid granules, so that the granular loci of the indicated activities is an artifact. This criticism is not applicable to the observed sequential color changes in Janus green B which are typical of the mitochondrion and occur in intracellular bacterial granules or, though more slowly, in free granules present in bacteriophage lysates. While, then, these bacterial granules may not be mitochondria, there is evidence that they have enzymatic activity.

The greater part of the enzymatic activity of the bacterial cell is associated with the submicroscopic granules, and with the soluble substances, *i.e.*, $<$ 20 S, present in the ground plasm. The particle size range is broad, although the bulk of the material occurs in the 40 S (10 to 20 mμ) fraction, but there appears to be no definite relation between size and localization of kinds of enzymatic activity. It is possible that the larger particles are aggregates, and the smaller particles fragments, of the 40 S fraction, *i.e.*, the activity of even microscopic granules may be in part a consequence of the presence of occluded submicroscopic particles, while sedimentable enzymatic activity may be solubilized by treatment of sediments with sonic oscillation. Within these implied reservations, kinds of enzymatic activity may be localized in sedimented particles and in supernatant which is assumed to represent ground plasm of the cell.

Components of the electron transport mechanism, including cytochromes and flavin pigments, cytochrome oxidase, DPN-cytochrome c reductase, and DPNH (reduced DPN) oxidase are present in large part, and possibly almost exclusively, in the submicroscopic particulates. The enzymes functional in the tricarboxylic cycle, however, are found to be divided between sedimentable particles and the ground substance of the cell. Of these, succinic dehydrogenase occurs almost exclusively in the granules, and 90 per cent or more of the total activity of the cell may be so accounted for. Malic dehydrogenase is found in both granules and supernatant, while aconitase and isocitric dehydrogenase occur exclusively in the supernatant. The localization of α-ketoglutaric dehydrogenase is uncertain. Pyruvic dehydrogenase is also found in the supernatant, but the system linking it to O_2 is in the granules. In the catalysis of some of the physiological activities, then, there is a synergistic relation between the granules and the ground plasm of the cell.

The submicroscopic granules emerge as biochemically complex structures capable of carrying out electron transport as well as the oxidation of organic acids at rates substantially equivalent to those reached by the intact cell. It follows that the components, including cofactors, of the catalytic systems are not only present, but present in physiologically available form, and the individual activities are oriented to give an organization having maximal activity. Thus the submicroscopic particle is an integral basic unit of the cell structure and organization, with a firmly established role in oxidative phosphorylation,[23] and is perhaps the bacterial equivalent of the mitochondrion of larger animal cells. Quantitative considerations suggest that the granules may be enzymatically heterogeneous. Assuming a diameter of 20 mμ, a specific gravity of 1.2, and protein to about one third of the wet weight, such a particle could contain about 14 protein molecules with an average molecular

weight of 35,000. It is difficult to reconcile such an estimate with the known presence of a complete electron transport mechanism and succinoxidase system. If there are enzymatically different kinds of granules, they are not separable by differential centrifugation.

COLONIAL MORPHOLOGY OF BACTERIA

When bacteria are grown on the surface of a nutrient medium solidified with agar or gelatin, the proliferating cells remain approximately fixed in position and form masses of many millions of cells that are visible to the naked eye. The colonies so formed range from a minute, barely visible size to masses several millimeters in diameter. They have characteristics, not only of size, but also shape and texture, and in some instances color, which, while dependent upon the nature of the culture medium and the conditions of incubation to an appreciable degree, under controlled conditions are constant and often of considerable differential value. Colonial morphology is, then, one of the morphological characteristics of bacteria, and one that is indispensable in primary isolation.

The size of bacterial colonies is, assuming favorable cultural conditions, quite uniform within species or type. Streptococcus colonies are, for example, relatively small, 1 mm. or less in diameter, as are those of most types of the closely related pneumococcus, while those of staphylococcus and enteric bacilli are somewhat larger, and those of Bacillus species may be several millimeters in diameter. Minute, dwarf, or D, colonies, much smaller than usually observed, may occur but represent a variant not usually encountered.

The shape of the colony is determined by its edge and by its thickness. The edge may be smooth, or it may be irregular and serrated to a greater or lesser degree. When the thickness is much greater in the center, diminishing uniformly to the edge, the colony is said to be raised, occasionally so much so as to approach a hemispherical form. Or it may be relatively uniform so that the colony appears to be little more than a disk on the surface of the medium.

The consistency and texture of the mass of cells are also distinguishing features of colonial morphology. The former ranges from the extreme of a dry, friable nature, such that when the colony is touched with a sterile wire it can be pushed about on the surface of the medium, to a viscous consistency so that

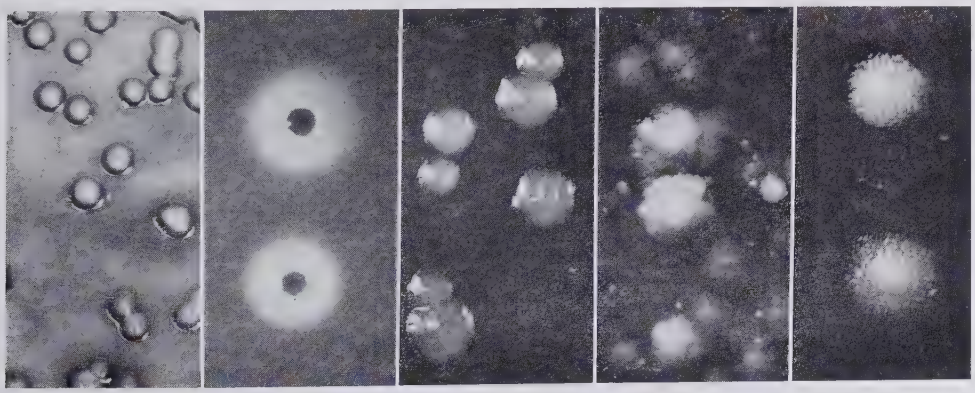


Figure 22. Morphological types of bacterial colonies. *From left to right,* the raised, smooth, viscous colonies of the gonococcus on chocolate agar; β-hemloytic streptococcus colonies on blood agar showing the cleared zones of hemolysis and the slightly matt, slightly irregular edge of the typical colony; colonies of the typhoid bacillus on nutrient agar, showing the typical irregular edge (maple leaf appearance) and irregular but smooth surface; colonies of the tubercle bacillus on Löwenstein's medium showing the characteristic roughened appearance; and colonies of the anthrax bacillus on nutrient agar in the typically rough, virulent form.

the cell mass clings to the wire with which it is touched, stringing away from the colony when it is withdrawn. The surface of the growth may be uniformly smooth and glistening, or it may be striated with indentations, radial or concentric, continuous or broken. On examination with transmitted light, the cell mass may appear as amorphous or granular in texture and varies from almost complete translucency, with perhaps a bluish cast, through varying degrees of opalescence to a white or yellowish opacity.

Pigmentation is more common among the saprophytic bacteria, and the cell mass may appear red, orange, yellow, etc., because of the presence of carotinoid pigments, and green in the case of some of the photosynthetic bacteria containing bacteriochlorin. Of the pathogens, the only important pigmented form is *Staphylococcus aureus,* whose colonies are a golden-yellow in color. Pigmentation is not apparent in the individual cells for the pigment occurs in intracellular granules too small to be resolved with visible light.

Since these characteristics occur in varying degree and combination from one kind of bacterium to another, colonial appearance is often quite characteristic, and kinds of bacteria may be differentiated from others in mixed or contaminated cultures. Differentiation on the basis of colonial morphology has hardly more than tentative status, however, and detailed study of the physiological and immunological characteristics of a bacterium is ordinarily required for identification.

Colonial morphology is analogous to a statistic in that it is derived from the individual cells but is a characteristic of the cell mass. Thus, pigmentation is apparent not in the individual cell but in the colony; the viscous consistency of the colony derives from the capsular substance of heavily encapsulated bacteria; the coiled texture of Bacillus colonies is a consequence of the tendency of the cells to remain attached end to end and form filaments; actively motile bacteria, such as Proteus, actually move or swarm over the surface of the medium to give a continuous film of growth, etc. In this way colonial morphology may be associated with other significant characteristics of the bacterium, for example, among the pathogenic bacteria virulence may be associated with capsule formation, and when this is true the colony of the virulent form is smooth in appearance and texture, and that of the avirulent form is rough (Chap. 7).

Colonial characteristics may be accentuated or induced by cultivation of the bacterium on so-called differential culture mediums which exploit relevant physiological properties. When bacteria are grown on an agar medium containing defibrinated blood, areas of greenish discoloration or clear zones in the red opaque medium appear around the colonies of bacteria which form hemolysins which lyse the erythrocytes and metabolize the hemoglobin to greenish or colorless compounds; such bacteria are said to be α- or β-hemolytic respectively. The ability of diphtheria bacilli to reduce tellurium salts gives rise to black colonies on tellurite differential mediums. Similarly, a sugar such as lactose may be incorporated in the medium together with an indicator such as Schiff's reagent or an acid-base indicator, so that the bacteria fermenting the sugar restore the color to the decolorized fuchsin and the colonies are red or are colored by the indicator because of the local concentration of acidity in the colony. Colonial morphology on such mediums is almost invariably a special case in that the medium is devised to enhance or induce a colonial appearance characteristic of the bacterium in question in order to facilitate its isolation from other bacteria in mixed culture.

THE MORPHOLOGY OF RICKETTSIAE AND VIRUSES

The microorganisms known as rickettsiae and viruses are set apart from the true bacteria, the Eubacteriales, on a physiological rather than a morphological basis. With rare exceptions, the bacteria are not primarily intracellular parasites, and are able to proliferate on lifeless nutrient mediums. In contrast, the rickettsiae and viruses grow only in close association with living host cells and are found within the cytoplasm or nucleus. While the largest of these forms link them in continuous series with the smallest bacteria, with progressive decrease in size these agents become distinct both morphologically and in chemical composition.

THE MORPHOLOGY OF RICKETTSIAE

The rickettsiae are morphologically similar to the smallest bacteria. They are cocco-bacillary to bacillary in form, 0.3 μ x 0.3 to 0.5 μ and occasionally as long as 2 μ, and overlap in size range minute bacteria such as *Pasteurella tularensis* and Bartonella. They are, therefore, demonstrable with the light microscope. They are uniformly gram-negative and stain poorly or not at all unless the dye solution is buffered and mordants are used. In smears and tissue sections they stain purple with Giemsa. In smears stained by Macchiavello's method (buffered fuchsin, citric acid solution, and methylene blue counterstain) the rickettsiae are red and tissue cells blue, and the buffered methylene blue–safranine counterstain method of Castaneda stains them light blue on a pink background. They also may be stained with diluted Wright's stain following methanol fixation.[104]

They occur singly and in pairs, and most commonly in dense intracellular masses, rickettsiae of the typhus group characteristically in the cytoplasm and those of the spotted fever group in the nucleus of the host cells, particularly those of mesothelial origin which line the serous cavities. They are nonmotile and do not form spores.

The protoplasm stains homogeneously, but Feulgen-positive granules similar to those found in bacteria occur, and both DNA and RNA are present in the cell. In electron micrographs a capsular outer layer, limiting membrane, and electron-dense granules are apparent. In thin sections of *Coxiella burnetii* the limiting membrane is 5 to 10 mμ thick, and appears to resemble closely the cell wall of bacteria in morphological and chemical properties.[5, 115] The cell contains a relatively large electron-dense body in

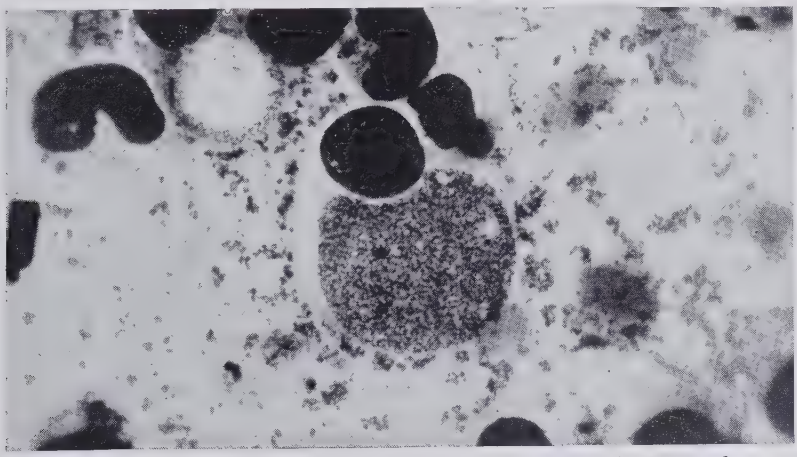


Figure 23. Rickettsiae of Q fever in a smear of skin exudate. Note the macrophage packed with enormous numbers of rickettsiae. × 1125.

addition to granules.[130] On treatment of typhus rickettsiae with ether to liberate soluble antigen, the capsular substance is removed and probably represents this antigen.

While the structure of the rickettsiae has not been subjected to as intensive study as has that of the bacterial cell, it seems clear that these microorganisms are closely similar to bacteria, and their physiological character is not reflected in significant differences in morphology. Since they are not cultivable on lifeless mediums, they have no colonial morphology to correspond to that of the bacteria, but the closely packed aggregates in which they occur within the host cell are suggestive of the viral inclusion body (see below).

While the rickettsiae are incapable of proliferation apart from the host cell, they contain both oxidative enzymes and transaminases. The typhus rickettsiae metabolize glutamate to oxalacetate by way of the scheme

glutamate → ketoglutarate → succinate → fumarate → malate → oxalacetate

and both this mechanism and that of transamination between glutamate and oxalacetate are closely similar to, if not identical with, those found in bacteria (Chap. 5) and higher animals, but these forms lack the enzymatic equipment necessary for independent existence.

THE MORPHOLOGY OF VIRUSES[10, 30, 31, 56, 63, 66, 97, 114, 119, 134, 143]

The group of agents known collectively as the viruses share the characteristics of small size and obligate parasitism but are otherwise heterogeneous. It is customary to separate them into three subgroups on the basis of host specificity, *viz.*, the bacterial viruses, the animal viruses, and the plant viruses. In some instances there is an association of morphological character and chemical composition with the host, or even disease, specificity, and some broad distinctions may be made.

Inclusion Bodies. Infection with many animal and plant viruses, but not bacterial viruses, results in the development within the host cell of masses of material, or inclusion bodies, demonstrable with the light microscope in stained preparations. Inclusion bodies

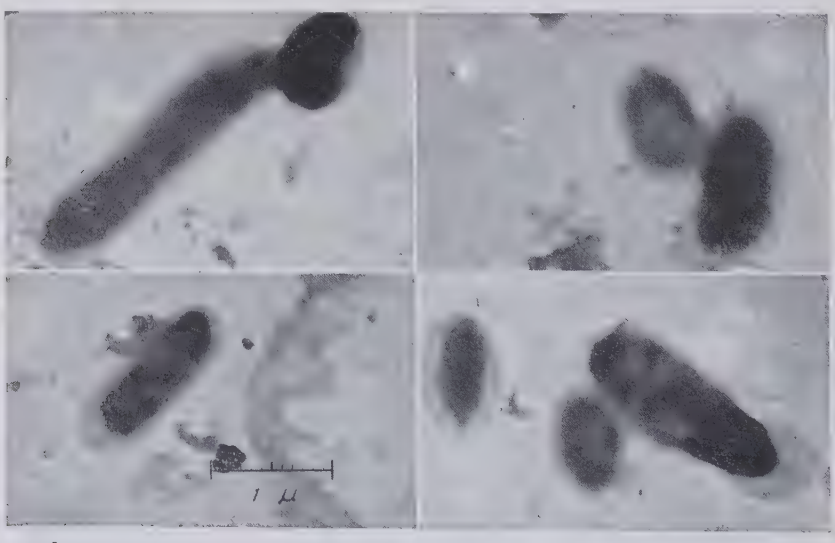


Figure 24. Electron micrographs of typhus rickettsiae from egg yolk culture. Note the variability in size, ranging from cocco-bacillary to bacillary forms, and the capsular material surrounding the individual cells. (Lilly Research Laboratories.)

are divided into two groups on the basis of their location within the cell. The inclusion bodies of the pox group of diseases (variola, vaccinia, fowl pox, etc.), myxomatosis, the psittacosis-lymphogranuloma group of diseases, etc., are found in the cytoplasm of the infected cell. Those associated with other virus diseases are characteristically intranuclear, and are of two kinds. One, designated type A, is a relatively large body which disrupts the structure of the nucleus and is found in cells infected with viruses such as herpes, varicella, and pseudorabies. The other, type B, found in a few virus diseases including poliomyelitis, is much smaller and occurs in an otherwise normal-appearing nucleus.

In some instances inclusion bodies have been named after the workers who described them, such as the Guarnieri bodies of variola and vaccinia, and the Negri bodies of rabies. Their presence contributes significantly to the characteristic histopathology of a number of virus diseases. For example, while the rabies virus produces inflammatory and degenerative changes in the central nervous system, especially in the spinal ganglia, the disease may be diagnosed with confidence only by the demonstration of Negri bodies present in the cytoplasm of the large ganglion cells in stained impression smears or tissue sections, preferably of the hippocampus major or cerebellum.*

In at least some instances cytoplasmic inclusion bodies are aggregates of infectious units or virus particles and may be considered as analogous to the bacterial colony. This was unequivocally demonstrated, first with fowl pox, in the now classic study of Woodruff and Goodpasture. When the inclusion body is teased apart, it breaks up into minute granules, 200 to 300 mμ in diameter in the case of the pox viruses and those of the psittacosis group, that may be shown to be the infectious unit of the virus. Various names have been given them in the past such as the Paschen bodies of variola and vaccinia, L.C.L.† or initial bodies of psittacosis, or, more generally, elementary bodies. Such terms, with the possible exception of the last, have tended to drop out of common usage, and the infectious virus unit is commonly called the virus particle, or simply virus.

In other virus diseases such aggregates of infectious units are not demonstrable, and the virus particles are liberated by dissolution of the host cell structure, or in the absence of degeneration are shed into the environment in some other way, in some instances possibly by a process of extrusion.

It is not established that all inclusion bodies found in virus diseases are aggregates of virus. On the contrary, the nature of many inclusion bodies, and intranuclear inclusions in particular, is open to question. Many do not have a characteristic morphology associated with the disease agent or occur in the apparent absence of virus disease such as liver cell inclusions appearing after severe burns. It is probable, in some instances at least, that they represent a cellular response to injury.

The Structure of the Virus Particle. Knowledge of the structure of the virus particle is largely confined to that obtained by chemical analysis of purified preparations of virus and electron microscopy of such preparations and of ultrathin sections of virus-infected tissue. The former may contain artifacts, both morphological and chemical, originating in the host cell substrate, though some chemical substances may not be artifacts in that host cell material incorporated rather than occluded in the virus may retain some of its original character. In addition, there is good reason to believe that the usual virus preparation is that of the agent in a resting rather than an

* Failure to find Negri bodies cannot be taken as evidence of the absence of infection with rabies virus. The laboratory diagnosis of this and other virus diseases is described specifically in connection with the diseases.

† So-called because they were described independently, and practically simultaneously (1930), by Levinthal in Germany, Coles in England, and Lillie in the United States.

actively reproducing state; for example, the interruption of intracellular virus synthesis results, in some instances, in "incomplete" virus (Chap. 4). Further, the necessary processes of drying, fixing, and embedding and cutting in the case of sectioned material, result in artifacts also. In general, then, information regarding the nature of the virus particle is subject to some degree of reservation.

Within the limitations so imposed—and with some readily purified viruses these are not as great as might be supposed—in general the virus particle, in the resting stage, consists of nucleic acid surrounded by a protective coat of protein. It is of interest that it was postulated by Crick and Watson[31] in 1956 that small viruses consist of viral nucleic acid surrounded by protein occurring as identical subunits, the subunits would occur as multiples of 12, and the virus particle would have cubic symmetry and tend to occur as polyhedrons. Of the polyhedrons, the icosahedron has the largest surface-volume ratio, *i.e.*, most closely approximates a sphere, and would have a cubic symmetry of 532. These predictions have been borne out for the small spheroid viruses,[63] but many of the larger viruses depart from the spheroid shape, and may, in fact, have a relatively complex structure. Three kinds of shapes, in addition to the spheroid, have been found to occur, *viz.*, a brick-shaped particle characteristic of the pox viruses, a rod-shaped particle such as certain of the plant and insect viruses, and a tailed spermlike or tadpole form which appears to be confined to the bacterial viruses. The relative sizes of these forms has been touched upon earlier, and it only need be noted here that the range is about twentyfold and that size is remarkably uniform within a single kind of virus, much more so than in a species of bacterium.

The occurrence of forms other than spherical in this size range is indicative of a very considerable degree of structural rigidity. This ranges from the relatively elaborate structure of the tadpole shape (see below) through the presence of a limiting membrane sufficiently rigid to maintain a hexahedral form, to the side-by-side orientation of elongated macromolecules. There is also evidence of an internal structure of the larger viruses which consists for the most part of the localization of electron-dense areas, possibly corresponding to concentrations of nucleic acid compounds, and the demonstration of the localization of DNA in the head of the tadpole-shaped bacterial viruses.

The viruses are also diverse in chemical composition. The larger forms, such as the pox viruses, contain protein, polysaccharide, and lipid in proportions similar to the composition of bacteria; vi-

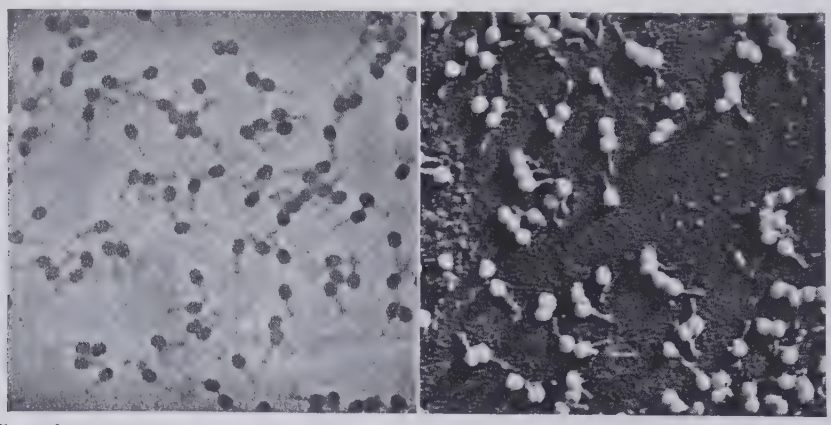


Figure 25. Electron micrographs of coliphage. The plate on the left shows untreated phage, and that on the right a similar preparation shadow-cast with chromium. (Sharp, Taylor, Hook and Beard.)

ruses of the influenza group, which are smaller, contain relatively more lipid; and the very small viruses, and some of the larger ones as well, are apparently pure nucleoprotein.

The nucleic acids of the bacterial viruses are of the DNA type exclusively and are present in large amount, up to 40 per cent, in the virus particle. The animal viruses may contain either DNA or RNA or both. In the plant viruses only RNA type of nucleic acid is found. In these last two groups the amount of nucleic acid present is variable from one virus to another, ranging from 6 to 30 to 35 per cent. The purine constituents are adenine and guanine, and the pyrimidines cytosine and uracil in the RNA type of nucleotide, and cytosine and thymine in the DNA type; it is of more than passing interest that in many of the bacterial viruses cytosine is replaced by hydroxymethyl cytosine. The adenine : thymine and guanine : cytosine ratios are usually close to unity. Intact virus particles are not broken down by nucleases, although the constituent proteins and nucleic acids are after separation, and the organization of the virus particle, like that of the living cell, effectively protects its constituents.

The Bacterial Viruses (Bacteriophages)[1, 7, 8, 52, 53, 54, 98, 127]

Transmissible lysis of bacteria was discovered by Twort in 1915 and rediscovered by d'Herelle in 1917 and for many years was known as the Twort-d'Herelle phenomenon. The agent is present in bacteria-free filtrates of lysed cultures of bacteria, and the addition of such filtrate to a fresh, growing culture results, after a suitable period of incubation, in lysis manifested as a clearing of a broth culture, or a cleared zone, or plaque, in a uniform film of bacterial growth on the surface of an agar culture medium. The agent was called bacteriophage by d'Herelle—frequently shortened to the diminutive, phage—and was consistently maintained by him to be a minute living agent parasitic on bacteria. It is now clear that bacteriophage is not a single agent adaptable to various kinds of bacteria, as d'Herelle had supposed, but there are in fact many kinds of phages more or less readily differentiable from one another so that the term bacteriophage has become a collective one. These agents resemble so closely the viruses causing diseases of animals and plants that they are regarded as bacterial viruses. The bacteriophages are widely distributed in nature and may be isolated by the inoculation of cultures of susceptible bacteria with cell-free filtrates of feces, sewage, polluted surface waters, etc., and, in addition, may also be found in many bacteria as a latent infection.

Since the bacterial viruses are readily manipulated in the laboratory, they have served as a prototype in the investigation of the nature of viruses, although it does not follow that they are directly comparable in all respects with animal and plant viruses. Consequently, they are better known than most of the other viruses, and of the bacterial viruses a group parasitic on coliform bacilli have been by far the most intensively investigated. There are seven of these "T" strains in all, of which T_2, T_4, and T_6, the "T-even" phages, constitute one serological group, T_3 and T_7 another, while T_1 and T_5 are unrelated to one another or the other T-coliphages. All of these are tadpole-shaped, as are other phages which have been examined critically, but the tails of T_3 and T_7 are very short. Phages parasitic on other bacteria, including staphylococci, streptococci, and mycobacteria, also appear to have this same general type of morphology.[20]

In electron micrographs of freeze-dried preparations of the T-phages, the heads are polyhedral, almost crystalline, in form and, when observed either parallel or perpendicular to the tail, have six relatively linear sides. The heads of the T-even phages are elongated hexagons, 65 x 95 $m\mu$, while the other T-phages have hexagonal heads, ranging in size

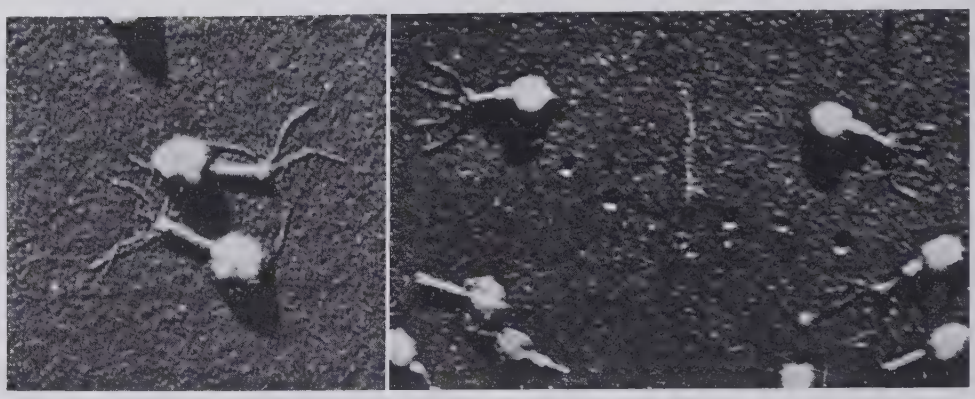


Figure 26. The structure of the distal portion of the tail of the T-even coliphages. *Left*, T_4 frozen and thawed to free the protein strands. × 100,000. (Williams.) *Right*, peroxide-treated T_2 showing the complete removal of distal tail protein and the protruding tail spike. × 100,000. (Kozloff.)

(diameter) from 47 mμ for T_3 and T_7, through 50 mμ for T_2 to 65 mμ for T_5. The tails vary in size and proportion; those of the T-even phages are 100 x 25 mμ, those of T_1 and T_5 are more slender, 150 x 10 mμ and 170 x 10 mμ respectively, while those of T_3 and T_7 are stubby and measure 15 x 10 mμ. The tail is not an organ of locomotion for it does not contribute to the rate of diffusion of the phage, *i.e.*, while the rate of adsorption on bacterial cells is proportional to the cell concentration, it is considerably less than the calculated collision rate and therefore not consistent with the assumption that the bacteriophages are actively motile.

Detailed study has made it clear that the phage particle is a differentiated, relatively complex structure. The head portion is made up of DNA, making up as much as 40 per cent of the virus particle as noted above, enclosed in an envelope or membrane of protein. The latter apparently has some function as an osmotic barrier for, when the particle is subjected to osmotic shock, the DNA leaks out to leave an empty shell.[37, 38]

The tail structure is also complex, the component parts playing different roles in the first stage of the reproductive cycle in which the phage particle is adsorbed at the tip of the tail onto the surface of the bacterial host cell (Chap. 4). The tail appears to be a tubular structure closed by a plug or tail spike. In the T-even and certain typhoid bacillus phages, the tail sheath is contractile, but in other phages it is not. The distal portion of the tubular structure is differentiated from the proximal portion in that the last few mμ are made up of

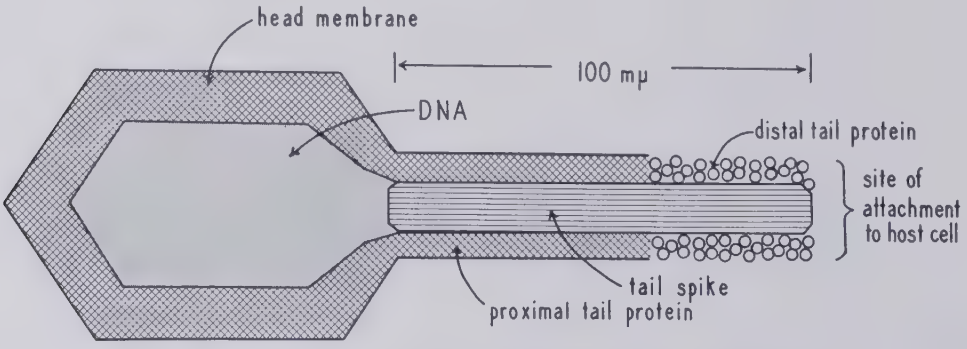


Figure 27. Schematic representation of the differentiated structure of coliphages of the T-even series

protein strands held together by a thiol ester linkage. It may be depolymerized by treatment with Zn^{++} or Cd^{++}, and is depolymerized following adsorption onto the host cell, in both instances leaving a portion of the tail spike uncovered. In addition, the extreme tip of the tail is differentiated, apparently with respect to the distribution of positively charged amino groups that play an essential part in the initial absorption.

Serological studies[69] have shown that at least two immunologically distinct proteins are present in the phage particle, the one in the head membrane and the other in the tail; antibody to the tail portion is that which neutralizes phage infectivity. It is clear that at least four distinguishable proteins are present, *viz.*, that of the head membrane, that of the proximal portion of the tail, that of the distal portion of the tail, and that of the tail spike.

The formation of areas of bacteriophage lysis in the confluent growth of susceptible bacteria on the surface of agar mediums has been noted above. When these are discrete, they are analogous to bacterial colonies in that it is a reasonable assumption that the area is a result of the proliferation of a single phage particle and consists of the progeny of that particle just as the bacterial colony is made up of the progeny of a single viable cell. Such discrete areas of phage lysis are known as plaques. Thus phages may be isolated in "pure culture" by streaking an agar medium previously inoculated sufficiently heavily with susceptible bacteria to give confluent bacterial growth, and the plaques may be picked and "subcultured." The occurrence of phage plaques also makes available an additional morphological character, that of plaque morphology.

While there are not as many variables, and therefore combinations of characteristics, in bacteriophage plaques as in bacterial colonies, the plaques have a distinctive character of differential value. There are, for example, "large plaque" phages, "small plaque" phages, and "minute plaque" phages. Another plaque type is the *tu*, or turbid halo, type characterized by a clear center surrounded by a diffuse cloudy zone. Or the margin of the plaque may be sharply defined or hazy. These plaque characteristics have the same validity as those of bacterial colonial morphology and may be regarded as phenotypic expressions of genetic character.

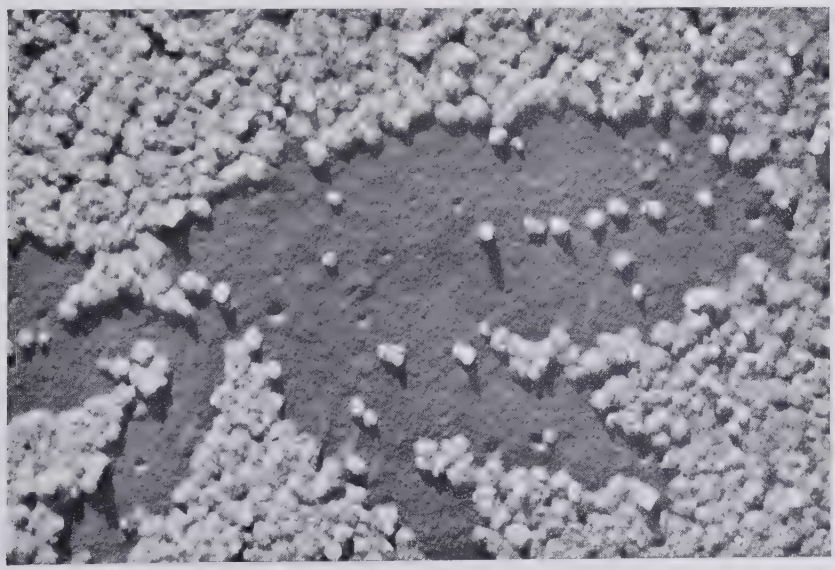


Figure 28. Electron micrograph of a shadow-cast preparation of a phage plaque left by the lysis of coliform bacilli by coliphage T_5. The granular texture of the fragments of bacterial protoplasm is clearly evident. × 19,440. (Wyckoff.)

Animal Viruses[11, 24, 116]

The animal viruses are more heterogeneous morphologically than the bacterial viruses, ranging in size from the large (300 to 400 mμ) psittacosis and pox viruses to some of the smallest (10 to 30 mμ) known viruses such as those of foot-and-mouth disease and poliomyelitis, and the group includes brick-shaped, rod-shaped, and spherical forms. Not all animal viruses have been studied in equal detail, but there is sufficient information available to give a reasonably accurate general picture of them, and some of the better known groups will be considered here.

The Psittacosis - Lymphogranuloma Group. The viruses making up this relatively homogeneous group include the causative agents of psittacosis or parrot fever, ornithosis, pneumonitis of man and lower animals, and the human venereal disease lymphogranuloma venereum. They are the largest of the animal viruses and are regarded by many as more closely related to the rickettsiae than to the other viruses, and some of the diseases they cause are, like rickettsial infections but unlike other virus diseases, susceptible to treatment with chemotherapeutic drugs. Of these agents, the psittacosis and lymphogranuloma venereum viruses are the best known.

The morphological character of these agents is complicated by the occurrence of virus particles of two sizes. One, the elementary body, is a sphere about 250 mμ in diameter. In electron micrographs these particles appear as collapsed sacs containing a dense central body apparent as a raised area in the flattened membrane when the preparation is shadowcast, and larger, 300 to 450 mμ in diameter, because of the flattening. These particles are taken to represent the infectious agent, probably a resting stage, although a single particle is not sufficient to produce infection.

On inoculation into susceptible tissue, larger bodies known as initial bodies appear after some hours. They are 650 to 800 mμ in diameter and are assumed to be swollen elementary bodies. The structure of these particles is unknown. Still larger bodies, sacs or vesicles, may be found in infected cells and tissues, but these have no distinctive morphology, and their relation to the virus is not completely clear.

The Pox Group of Viruses. The viruses making up the pox group are relatively homogeneous morphologically. The group includes the causative agents of diseases of man such as variola, vaccinia, molluscum contagiosum, herpes zoster, and chickenpox, and diseases of lower animals such as myxoma and fibroma of rabbits, fowl plague, and mouse pox.

The virus particles are brick-shaped and relatively large, 200 x 300 mμ, though some may be somewhat smaller. In electron micrographs a dense central portion is apparent. Digestion with pepsin accentuates the appearance of the central body and shows a limiting membrane. Subsequent treatment with deoxyribonuclease results in the dissolution of the central body, leaving the virus particle as an empty membrane. The structural similarities to the bacteria, *i.e.*, a central mass of DNA surrounded by "viroplasm" and enclosed within a single or double limiting membrane, have been noted by many workers.

While even the larger viruses are incapable of independent proliferation, there is some evidence suggesting that some enzymatic activity may persist. The susceptibility of infections caused by viruses of the psittacosis group to treatment with antibiotics such as chloramphenicol and the tetracyclines, as noted above, might be taken to suggest some small, but essential, metabolic activity or extracellular growth at some stage. In the case of the pox viruses, traces of riboflavin and biotin are found, together with esterase and nuclease activity. Whether these represent contamination with tissue constituents not removed by purification procedures, incidental incorporation of tissue constituents in the virus substance, or integral parts of the virus structure, is not known.

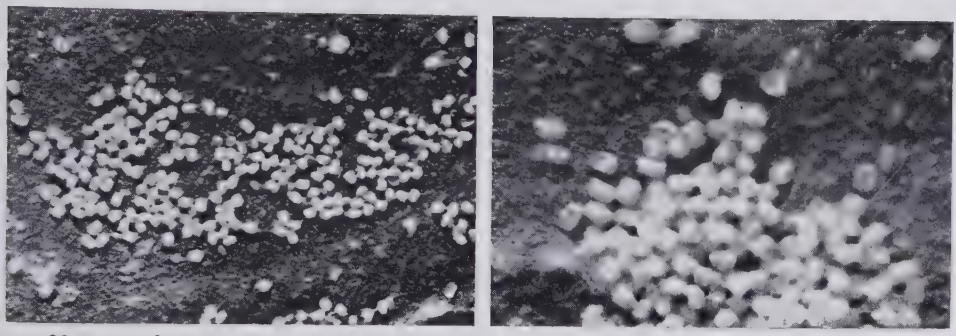


Figure 29. An electron micrograph of thin section through the chorioallantoic membrane of a 10-day chicken embryo 24 hours after inoculation with vaccinia. The numerous elementary bodies can be seen to possess a cuboidal or cylindrical shape. *Left,* × 4500; *right,* × 12,500. (Wyckoff.)

The Spherical Viruses. The spherical shape of the psittacosis and related viruses has been noted above, but many of the smaller animal viruses are also spherical or approximately spherical. In electron micrographs some of these agents may show flattened sides with an apparent tendency to a polyhedral form, and the surfaces may appear roughened. It is open to question to what extent these deviations from a smooth spherical form are artifacts.

In general, three groups of spheroid animal viruses are distinguished, largely on the basis of the tissues involved in the naturally occurring disease and the cellular response to infection. These are the so-called influenza-mumps-Newcastle disease (NDV) group; the tumor viruses producing benign or malignant hyperplasias, and including the Rous chicken sarcoma virus and human papilloma or warts virus; and finally, the viruses causing disease of the central nervous system, including the arthropod-borne encephalitides (equine encephalomyelitis, etc.), and the poliomyelitis and Coxsackie viruses, and Theiler's virus.

The influenza group.[16, 59, 90, 94] These viruses all occur as spheres and are relatively large, 70 to 175 mμ in diameter. Various deviations from this shape occur. Influenza virus, particularly recently isolated strains, shows the presence of filaments when cultured in the allantoic cavity of the embryonated hen's egg, and similar filaments are also observed with fowl plague virus. In addition, so-called incomplete virus may be formed under appropriate conditions. These morphological elements occur in connection with active proliferation of the virus and are discussed elsewhere (Chap. 4) in connection with growth. The occurrence of such forms tends to substantiate the assumption that virus morphology tends to be that of a resting stage.

The spherical influenza virus particle is 60 to 70 mμ in diameter. Those particles cut centrally in ultrathin sections of infected chorioallantoic membrane show a spherical internal body 20 to 22 mμ in diameter, a sharply defined limiting membrane 3 mμ thick, and an external layer of less dense, and possibly more diffuse, material.

The particles of NDV tend to be varied in morphology, with comma-shaped streamers attached to the particle. This indicates a plasticity, or lack of a structure as rigid as that of some other viruses, for such alterations from the spherical shape may be produced by varying salt concentrations, while formalin-fixed virus particles retain the spherical shape. Mumps virus has a similar tendency to show pseudopodia-like processes extruding from the periphery of the particle, but not as marked as that of NDV. Substantially nothing is known of the internal structure of the virus particles of this group.[139]

Viral Hemagglutination.[6, 26, 46, 139] The agglutination of red blood cells by viruses is not uncommon, and three groups of viruses may be distinguished, *viz.,* (1)

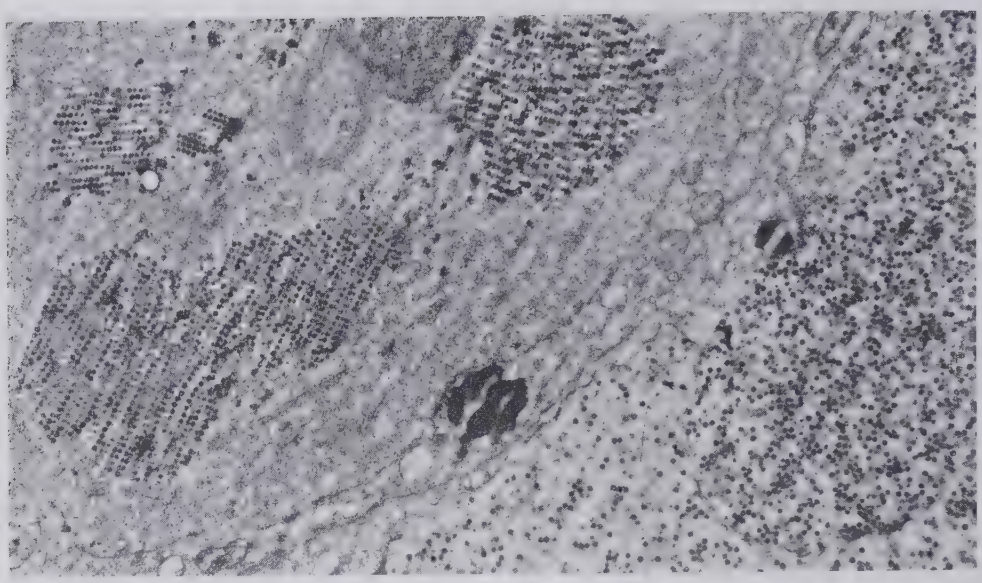


Figure 30. Adenovirus in two adjacent cells. In the cell on the right the virus particles are dispersed at random, but in the cell on the left they occur in ordered arrays, and the latter aggregates may be seen with the light microscope within infected cells. The two kinds of virus particles, the one more electron-dense than the other, are clearly apparent. × 11,550. (Councilman Morgan.)

the myxovirus group (Chap. 38), including the influenza and related viruses, in which (a) the virus particle is the agglutinating agent, and (b) the virus contains an enzyme which degrades the host cell receptor substance, and allows elution; (2) the arthropod-borne viruses (Chap. 40) and certain other neurotropic viruses in which the virus particle is the agglutinating agent, but there is no destruction of cell receptors and no spontaneous elution; and (3) the viruses, including certain of the pox group (Chap. 37) and the psittacosis group (Chap. 36), which produce a soluble hemagglutinin during growth. Such hemagglutination is usually specifically inhibited by homologous antibody, and the hemagglutination-inhibition test is a valuable serological method for the study of such viruses.

Of these, the myxovirus hemagglutination is the best understood of the viral hemagglutinations. When virus suspension is mixed with red cells, commonly chicken cells, and allowed to stand at room temperature to allow the cells to settle out, the cells adsorb the virus and are agglutinated, *i.e.*, when they settle out in the bottom of the tube they form a diffuse layer rather than a compact button. When virus is tested in serial dilution, a hemagglutinin titer may be measured which, in the case of fresh virus, closely parallels infectivity. When the agglutinated cells are allowed to stand for some hours, the adsorbed virus is spontaneously eluted, and the cells are no longer agglutinable with fresh virus. This is due to destruction of the receptor substrate with which the virus combines by enzymatic activity of the virus.

The enzymatic activity which destroys the myxoviral agglutinability of red cells is formed by a number of bacteria, notably *Clostridium welchii* and *Vibrio cholerae*. Cholera vibrio culture filtrates are now the conventional source of this receptor-destroying enzyme, or RDE. Viral hemagglutination is also inhibited by various nonspecific inhibitors which are often present in fluids, such as serum, which may contain antibody. Such nonspecific inhibitors are destroyed by pretreatment with RDE before testing for antibody activity by the hemagglutination inhibition test.

The cell receptor substance involved in myxovirus hemagglutination is a mucoprotein, protein conjugated with oligo-

saccharides. The size of the oligosaccharide groups and the number of them attached to the protein vary. The terminal units of the oligosaccharides are acetylated neuraminic acid residues, attached by a glycosidic linkage to the adjacent sugar. It is this linkage which is destroyed by RDE, or neuraminidase, with the consequent breakdown of the orderly arrangement of groups apparently necessary for adsorption of the virus. Such neuraminidase is apparently present in influenza and related viruses; its activity accounts for destruction of the cell receptor and consequent elution of adsorbed virus.

The myxoviruses are not identical in that, while red cells treated with one virus are no longer agglutinated by or adsorb that virus, they may or may not adsorb other viruses of the group and be agglutinated by them. This has led to the formulation of a receptor gradient series: approximately mumps-NDV-influenza A-swine influenza-influenza-B, in which red cells treated with mumps virus will still agglutinate in the presence of NDV and the influenza viruses; those treated with NDV will not be agglutinated by mumps virus or NDV but will be agglutinated by the influenza viruses; etc. This relationship is not absolute but dependent upon the amount of virus used and the length of time it is allowed to act upon the red cell. It is of interest that influenza C is unrelated to the other members of the group in this respect.

The tumor viruses. In addition to the myxoma and fibroma viruses, a number of spherical viruses also produce a hyperplasia rather than degenerative changes in infected tissue. The best known of these is the Rous chicken sarcoma virus, but the structure of the virus particle is not known other than it appears to be a sphere 60 to 70 mμ in diameter. Shope papilloma virus, infecting rabbits, is also a spherical particle, but smaller, about 40 mμ in diameter. Electron micrographs of preparations treated with uranyl nitrate as an electron stain show a number of small dense regions, 8 mμ in size, within the particle, but their significance is not known. Characteristic spherical particles about 50 mμ in diameter are found in human papillomas (warts). In ultrathin sections they occur as closely packed intranuclear or intracytoplasmic masses in the cells of the neoplastic epidermis, with almost a crystalline appearance because of the regular arrangement of the particles. Larger particles, 120 mμ in diameter and subject to saline distortion like NDV particles, are the etiological agents of at least one of the avian leucosis complex of diseases, and are found in the plasma of infected chickens. The infectivity of these particles is correlated with the ATP-ase activity of the purified preparations, but it is not known whether the enzymatic activity is an integral part of the virus.

The neurotropic group. Many, though not all, of the viral agents producing disease of the central nervous system are relatively small spheres, 50 mμ or less in diameter. One of the more obvious exceptions is rabies virus, which is larger and estimated at 125 mμ in diameter. Thus, the equine encephalomyelitis viruses are about 50 mμ in diameter, and there is evidence that the eastern variety (EEE) is a little smaller than the western variety (WEE). The virus of lymphocytic choriomeningitis is also about 50 mμ in diameter. The serologically related St. Louis encephalitis virus, Japanese B encephalitis virus, and West Nile virus are all smaller, 20 to 25 mμ in diameter, and that of louping ill (sheep) is of this same size.

The poliomyelitis-like group of viruses is made up of strains of the three serological types of poliomyelitis virus, differing in their adaptability to nonhuman host cells, and the multiplicity of agents known as the Coxsackie viruses that cause nonparalytic disease similar to mild poliomyelitis as well as other clinical kinds of disease. These are all small viruses, 25 to 30 mμ in diameter. It is of particular interest that two have been prepared in crystalline form.

Although the first virus to be crystallized was a plant virus, that of tobacco mosaic disease, which was crystallized in 1935 (see below), it was 20 years before

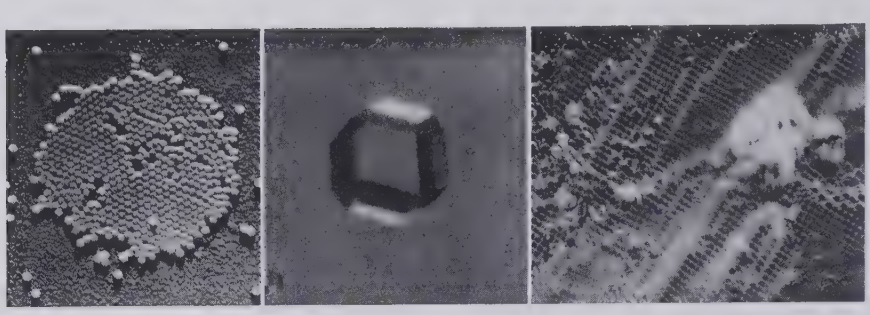


Figure 31. Poliovirus. *Left,* purified virus, showing the spherical form and tendency of the virus particles to pack together. × 8000. *Center,* a crystal of poliovirus. × 100. *Right,* the sheared surface of a crystal of the virus, showing the occurrence of the virus particles in orderly arrays. (Schwerdt.)

the first animal virus was prepared in this form. The MEF-1 strain of poliomyelitis virus from infected monkey kidney tissue culture was purified as a homogeneous component consisting of spheres 27 mμ in diameter, and this material crystallized from saline in the cold to give bipyramidal tetragonal prisms about 30 μ long and consisting of nucleoprotein.[117] A strain of Coxsackie virus (type 10) was purified by similar procedures from the whole carcasses of infected suckling mice, and crystallized from saline in the cold as small dodecahedrons, and from 5 per cent ammonium acetate as rectangular plates, both of which consisted of regularly arrayed particles 28 mμ in diameter. Both types of Coxsackie virus crystals were unstable and contained considerable inactive material, with a ratio of infectious to total particles of about 1:1000.[79]

The Insect Viruses.[14, 15, 121, 122, 124, 125, 126] A number of infectious diseases of insects, especially insect larvae, are caused by viruses. The majority of these diseases are characterized by the presence of inclusion bodies in the infected cells, which are made up of virus particles. Sacbrood of bees is exceptional in that inclusion bodies are not found.

Many of these inclusion bodies are polyhedral in shape, ranging in size from 0.5 to 15 μ, but most commonly 3 to 5 μ, and the diseases in which they occur are known as polyhedral disease or polyhedrosis. The best known of these is jaundice of silkworm (*Bombyx mori*) larvae and has been recognized since the early sixteenth century. Other insect larvae infected by similar agents are the nun moth, the gypsy moth, and some species of caterpillars.

The other kind of inclusion body is a small oval body, usually less than 1 μ in length, and the associated viruses are sometimes known as the capsule viruses or granulosis viruses. They produce disease in certain caterpillars, cutworms, and butterfly larvae.

The intact inclusion bodies are insoluble in water and are not affected by the processes of bacterial decomposition of the host tissue, so they may be prepared in relatively pure form by centrifugation. They may be dissolved in dilute alkali to liberate the virus particles, and presumably an analogous process occurs in the gut of the insect at the initiation of infection. The inclusion bodies consist of nucleoprotein (DNA type) virus particles embedded in a matrix of protein, which is serologically related to that of the virus particles, and enclosed in a "limiting membrane" of precipitated specific, or closely related, protein. The protein matrix makes up about 95 per cent, and the virus particles 3 to 5 per cent, of the inclusion body.

The particles of the majority of the insect viruses are rod-shaped, 20 to 27 x 200 to 400 mμ. It was believed for many years that all the insect viruses were rod-shaped until it was shown that in some instances spherical forms, 20 to 40 mμ in diameter, may predominate.

The rod-shaped particles occur in the inclusion body in small aggregates enclosed in a membrane. In the polyhedral inclusion body there may be 6 to 8 indi-

vidual rods arranged as bundles, while in the smaller oval inclusions single rods predominate, and occasionally two or three may be found within a single membrane. In the intact or disintegrating inclusion body, spheres may be observed occasionally, and there is reason to believe that the rods originate in the spherical bodies, which break down to liberate spheres in a reproductive cycle. Filaments may occur also which are reminiscent of those associated with some of the animal viruses. In its early stages the inclusion body is liquid in consistency, but it becomes more viscous as the protein concentration increases, and finally protein precipitates at the periphery to complete formation of the mature inclusion. In any case, it is probable that the rods represent the resting stage of the majority of these viruses, but in some instances the sphere may be the resting stage.

Plant Viruses[76]

A considerable number of diseases of plants are of virus etiology, and the third group of viruses is made up of these agents, the plant viruses, separable into more than 100 kinds. The diseases they produce include the yellows group of diseases (such as aster yellows); the mosaic diseases of tobacco and acuba; the ring spot diseases of tobacco, potato, and delphinium; the leaf-curl diseases of tobacco, cotton, and sugar beets; the leaf savoy (leaf deformation) diseases of beets and rutabaga; and spotted wilt of tomato.

Almost all of these agents are transmitted by arthropod vectors of the families Aphididae (aphids), Cicadellideae (leaf hoppers), Aleyrodideae (white flies), Coccideae (mealybugs), and Thrysanoptera (thrips). Of these vectors, the aphids are by far the most common, the leaf hoppers less so, and only one plant virus, that of spotted wilt, is transmitted by thrips; the white fly vectors are more common in tropical climates than in temperate climates. While transmission may be mechanical, and usually is for example in aphids, it may also be biological in that the insect vector is infected, is capable of transmitting the virus only after an incubation period, and may remain infectious for long periods or even for life. In a few instances, such as rice stunt virus, the virus is transmitted from the female to the egg.[75] Such biological transmission is of particular interest in that it indicates that the range of hosts capable of supporting growth of the

Figure 32. The virus of polyhedral disease of the gypsy moth (*Porthetria dispar*), showing the polyhedral body on the left. The "packets" of rod-shaped virus particles are enclosed within limiting membranes both within the polyhedral inclusion body and outside it, and the rod-shaped particles are those of mature free virus. × 22,000. (Bergold.)

virus extends from the plant to animal kingdoms. A number of these viruses have been cultivated in tissue cultures which simulate the insect tissues.[47]

Some of these viruses have been studied intensively, while others are not so well known. In general, they occur as two morphological types, the one rod-shaped and relatively large and including tobacco mosaic virus (TMV) and cucumber virus, both 300 x 15 mμ, and the virus of potato-X disease which is a somewhat longer, more slender rod, 430 x 10 mμ. The remainder of the plant viruses are small spherical or polyhedral particles 15 to 30 mμ in diameter. A number of these viruses, notably TMV and cucumber viruses, and the viruses of bushy stunt of tomato, tobacco necrosis, Southern bean mosaic, and turnip yellow necrosis, have been prepared in crystalline form. These viruses appear to consist of pure, or nearly pure, nucleoprotein. The nucleic acids of the plant viruses are of the RNA type exclusively, in contrast to the bacterial and animal viruses, with nucleic acid contents ranging from 5 to 6 per cent to as high as 40 per cent in the case of tobacco ring-spot virus. Two of the plant viruses, TMV and bushy stunt virus, may be taken, for present purposes, as examples of the better known plant viruses.

Tobacco Mosaic Virus.[96, 123] This virus, the first to be crystallized, was prepared by Stanley in 1934 as needle-shaped paracrystals. It is a pure nucleoprotein, containing 94 per cent protein and 6 per cent nucleic acid of the RNA type, with a molecular weight of about 5 million.

The infectious unit is a cylinder of protein, 300 x 15 mμ, with a core of nucleic acid, probably multistranded, about 4 mμ in diameter. The virus may be found in, or broken down to, units of less than this length, and may occur in multiples of it, but the smaller rods do not have virus activity. The rods are deeply grooved and may be packed screw-wise more tightly than the diameter of 15 mμ would suggest. The way in which the nucleic acid and protein components are bound is not known, but it is of such a nature that the nucleic acid is not attacked by RNA-ase in the intact particle.

The particle may be broken up by

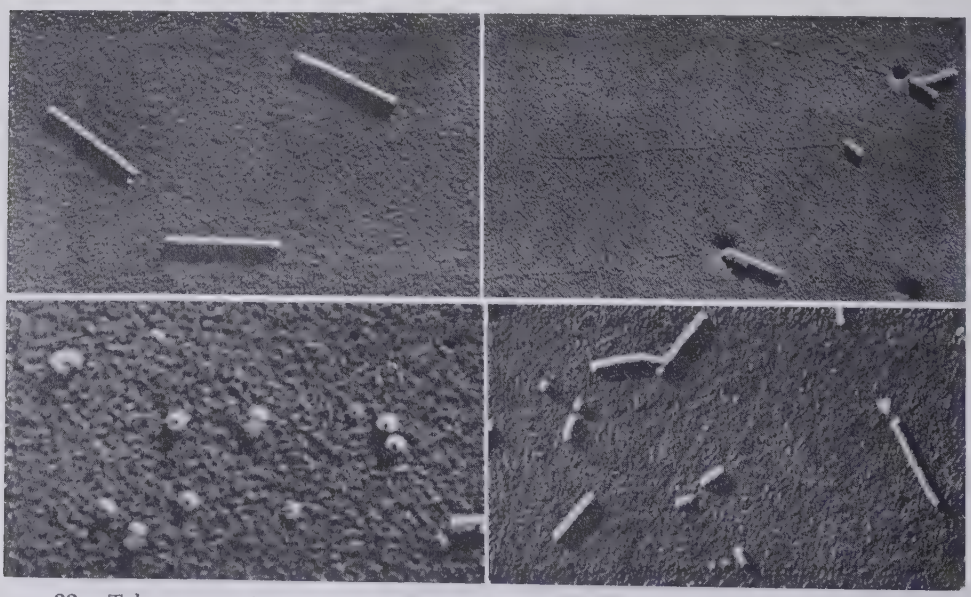


Figure 33. Tobacco mosaic virus. *Upper left,* the infectious particle of the virus. × 58,000. *Upper right,* partially disintegrated virus particles, showing the fine strands of the central core of nucleic acid attached to the fragments. × 42,000. *Lower left,* the washer-shaped units of the cylindrical protein structure. × 115,000. *Lower right,* "reconstituted" virus, showing the variability in size of the particles so produced. × 50,000. (Williams.)

treatment with alkali, sodium dodecyl sulfate, or urea, with separation of protein and nucleic acid, and disintegration of the protein into chemical subunits with a molecular weight of 17,000 to 18,000 in which threonine is the C-terminal amino acid, and the N-terminal is bound in a ring structure. The peptide chain of 145 residues is coiled in the form of a helix so that the subunits have a washer-like form. The minimum size of subunit that can be polymerized to give a rod-shaped structure is, however, larger than this, with a molecular weight of about 100,000.

It has been possible to copolymerize the protein and nucleic and components after separation in sodium dodecyl sulfate to give rods of the original composition and size and having viral activity. Further, a "hybrid" virus has been prepared by combining nucleic acid from the ribgrass strain of TMV with protein from ordinary TMV to give a virus that produced a disease characteristic of the ribgrass strain and whose progeny contained both ribgrass protein and nucleic acid. The relatively greater importance of the nucleic acid portion of the molecule is further indicated by the production of relatively extensive changes in the protein without affecting viral activity. Thus, activity is not lost when as much as 70 per cent of the free amino groups are acetylated, or all the -SH groups are reacted with iodine, though inactivation occurs when iodine is substituted in the tyrosine ring. Again, the progeny of such altered virus contain the normal TMV protein component. It is clear that such studies are beginning to approach the goal of some understand-

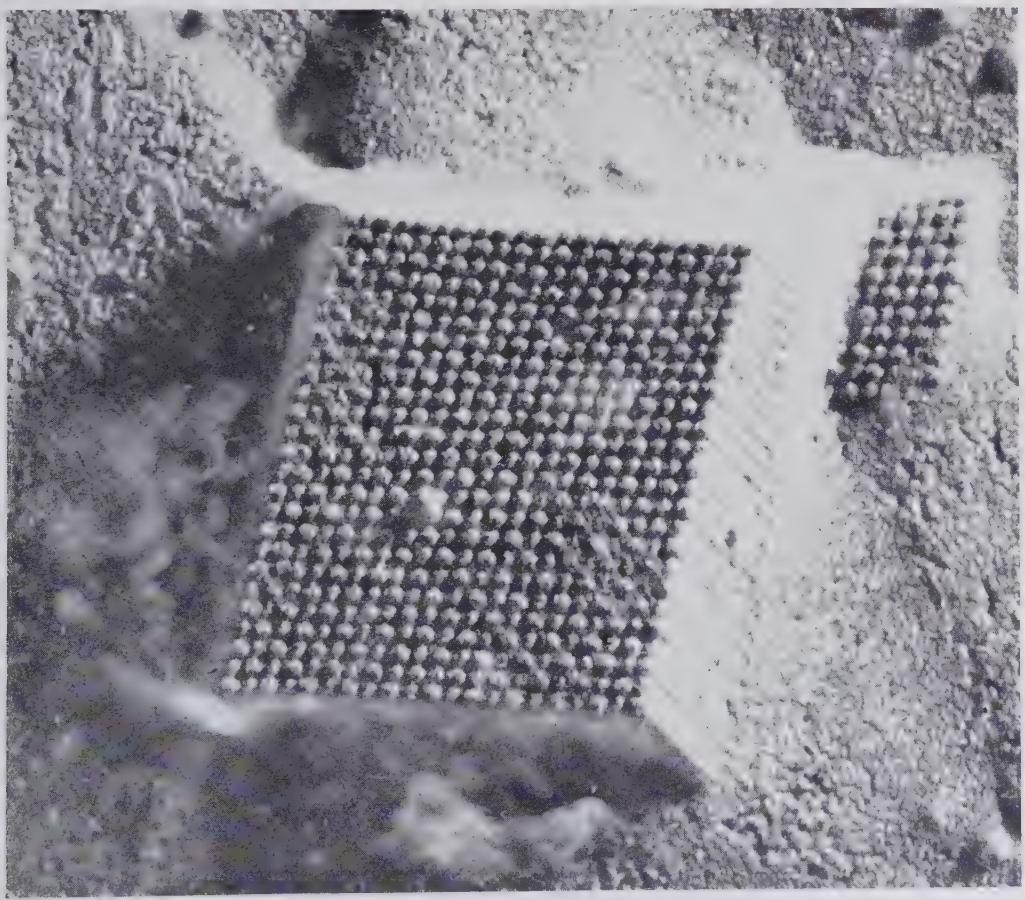


Figure 34. Electron micrograph of an unusually perfect rhombic type crystal of tobacco necrosis virus, prepared by the carbon replica technique. × 42,000. (Labaw and Wyckoff.[68])

ing of the nature of viral, or for that matter protoplasmic, replication during the processes of growth.

Bushy Stunt Virus.[27] The virus causing bushy stunt of tomato is representative of the smaller spherical plant viruses. It has been crystallized in the form of dodecahedral crystals and is apparently largely, if not entirely, nucleoprotein, but contains considerably more, 16 per cent, nucleic acid than TMV. In electron micrographs of air-dried preparations it appears to be spherical in shape and about 30 mμ in diameter. In freeze-dried preparations, it is apparent that the virus particles are polyhedral rather than spheroid in shape and in cross section are usually hexagonal. The virus particles tend to occur in orderly arrays, and the crystals are made up of such arrayed particles. X-ray diffraction studies suggest that the virus particle is made up of 60 subunits with a molecular weight around 125,000.

COMPARATIVE MORPHOLOGY OF MICROORGANISMS

The fungi and animal parasites considered elsewhere are more complex and morphologically conventional than the bacteria in that even the unicellular forms are differentiated into the usual nucleus and other intracellular structures. Beginning with the bacteria, and the blue-green algae not touched upon here, this familiar pattern of cell structure begins to dissolve.

Among the bacteria the existence of a functional, morphologically discrete nucleus becomes nebulous, and its place is perhaps taken by the chromatinic body, containing large amounts of DNA but of no more than equivocal relation to a nucleus. Polyphosphate-containing granules visible with the light microscope occur, but apparently function in the storage of energy in the form of high-energy phosphate bonds, and do not have the degree of physiological competence associated with the mitochondrion of conventional cells. Rather, mitochondrial function is associated in part with minute intracellular particulates, and quantitative considerations suggest that even these, as individual particles, may not have the metabolic activity they show in the aggregate. The cell wall persists as a supporting structure and may, because of its minor contribution to the osmotic barrier, have survival value, but it may be dispensed with to leave a functional physiological unit, the bacterial protoplast, apparently lacking only the ability to initiate spore formation.

In the size range of the larger viruses the inevitable effects of size become even more apparent in that the differentiated structure that persists serves to house and protect a nucleic acid directive mechanism, and the unit consists of little more than a protective membrane surrounding an electron-dense body consisting, presumably, largely of nucleic acid compounds. Finally, in the simplest viruses there is little or no structure, and the particle consists of naked nucleoprotein that has organizational individuality only indirectly through the directive mechanism it contains and possibly can be considered as "living" only because it can reproduce itself indefinitely in series in a host cell. It is probable that the "living organization" reaches its lower limit at the dimensions of large protein molecules; thus the smallest subunit of TMV that can be polymerized to give an infectious unit has a molecular weight of 100,000, and the subunit of bushy stunt virus has a molecular weight of 125,000. This apparent lower limit in size may be taken to represent that in which the nucleic acid is effectively protected by the protein coat, *i.e.*, that of resting virus. With the demonstration of infectious nucleic acid (Chap. 4), the lower limit may be reduced to that of the size of a macromolecule of polymerized nucleic acid.

REFERENCES

1. Adams, M. H.: Bacteriophages. Interscience, New York. 1959.
2. Adler, H. I., and Zelle, M. R.: Quantitative evaluation of the effects of various methods of fixation on the appearance of stained

structures in the bacterial cell. J. Bact., 1957, **73**:526–529.
3. Albertsson, P-A.: Partition of Cell Particles and Macromolecules. John Wiley and Sons, New York. 1960.
4. Alexander, M.: Localization of enzymes in the microbial cell. Bact. Rev., 1956, **20**:67–93.
5. Allison, A. C., and Perkins, H. R.: Presence of cell walls like those of bacteria in rickettsiae. Nature, 1960, **188**:796–798.
6. Anderson, S. G.: Hemagglutination by animal viruses. In Burnet, F. M., and Stanley, W. M. (eds.): The Viruses, vol. 3, pp. 21–50. Academic Press, New York. 1959.
7. Anderson, T. F.: The morphology and osmotic properties of bacteriophage systems. Cold Spring Harbor Symposia Quant. Biol., 1953, **18**:197–203.
8. Anderson, T. F.: Bacterial viruses—structure and function. In Gunsalus, I. C., and Stanier, R. Y. (eds.): The Bacteria, vol. I, pp. 387–414. Academic Press, New York. 1960.
9. Astbury, W. T., Brighton, E., and Weibull, C.: The structure of bacterial flagella. Society for Experimental Biology, Symposium No. 9, pp. 282–305. Academic Press, New York. 1959.
10. Bang, F. B.: Morphology of viruses. Ann. Rev. Microbiol., 1955, **9**:21–44.
11. Bang, F. B.: The morphological approach. In Burnet, F. M., and Stanley, W. M. (eds.): The Viruses, vol. 3, pp. 63–110. Academic Press, New York. 1959.
12. Bartholemew, J. W., and Mittwer, T.: The Gram stain. Bact. Rev., 1952, **16**:1–29.
13. Bennett, A. H., Jupnik, H., Osterberg, H., and Richards, O. W.: Phase Microscopy. John Wiley and Sons, New York. 1951.
14. Bergold, G. H.: Insect viruses. Adv. Virus Res., 1953, **1**:91–139.
15. Bergold, G. H.: Biochemistry of insect viruses. In Burnet, F. M., and Stanley, W. M. (eds.): The Viruses, vol. 1, pp. 505–524. Academic Press, New York. 1959.
16. Birch-Anderson, A., and Pauker, K.: Studies on the structure of influenza viruses. II. Ultrathin sections of infectious and noninfectious particles. Virology, 1959, **8**:21–40.
17. Bisset, K. A.: Do bacteria have mitotic spindles, fusion tubes and mitochondria? J. Gen. Microbiol., 1953, **8**:50–57.
18. Bowen, T. J., Dagley, S., Sykes, J., and Wild, D. G.: Nature of the ribosomes present in bacteria and yeasts: A reappraisal. Nature, 1961, **189**:638–640.
19. Bradfield, J. R. G.: Organization of bacterial cytoplasm. Society for General Microbiology, Symposium No. 6, pp. 296–317. Cambridge University Press. 1956.
20. Bradley, D. E., and Kay, D.: The fine structure of bacteriophages. J. Gen. Microbiol., 1960, **23**:553–563.
21. Brenner, S., et al.: Bacterial protoplasts. Nature, 1958, **181**:1713–1715.
22. Brinton, C. C.: Non-flagellar appendages of bacteria. Nature, 1959, **183**:782–786.
23. Brodie, A. F., and Gray, C. T.: Bacterial particles in oxidative phosphorylation. Science, 1957, **125**:534–537.
24. Burnet, F. M.: Animal viruses: A comparative survey. In Burnet, F. M., and Stanley, W. M. (eds.): The Viruses, vol. 3, pp. 1–14. Academic Press, New York. 1959.
25. Burton, E. F., and Kohl, W. H.: The Electron Microscope: Its Fundamental Principles and Application. Reinhold, New York. 2nd ed., 1946.
26. Buzzell, A., and Hanig, M.: The mechanism of hemagglutination by influenza virus. Adv. Virus Res., 1958, **5**:290–346.
27. Caspar, D. L. D.: The structure of bushy stunt virus. Nature, 1956, **177**:475–476.
28. Clark, G. L. (ed.): The Encyclopedia of Microscopy. Reinhold, New York. 1961.
29. Cohn, G. N., and Monod, J.: Bacterial permeases. Bact. Rev., 1957, **21**:169–194.
30. Colter, J. S., and Ellem, K. A. O.: Structure of viruses. Ann. Rev. Microbiol., 1961, **15**:219–244.
31. Crick, F. H. C., and Watson, J. D.: Structure of small viruses. Nature, 1956, **177**:473–475.
32. De Lamater, E. D.: Cytology of bacteria. II. The bacterial nucleus. Ann. Rev. Microbiol., 1954, **8**:23–46.
33. Doak, B. W., and Lamanna, C.: On the antigenic structure of the bacterial spore. J. Bact., 1948, **55**:373–380.
34. Duguid, J. P.: The demonstration of bacterial capsules and slime. J. Path. Bact., 1951, **63**:673–685.
35. Duguid, J. P., and Gillies, R. R.: Fimbriae and adhesive properties in dysentery bacilli. J. Path. Bact., 1957, **74**:397–411.
36. Duguid, J. P., Smith, I. W., Dempster, G., and Edmunds, P. M.: Non-flagellar filamentous appendages ("fimbriae") and hemagglutinating activity in *Bacterium coli*. J. Path. Bact., 1955, **70**:335–348.
37. Evans, E. A., Jr.: Bacteriophage as nucleoprotein. Fed. Proc., 1956, **15**:827–832.
38. Evans, E. A., Jr.: The comparative chemistry of infective virus particles and their functional activity: T-2 and other bacterial viruses. In Burnet, F. M., and Stanley, W. M. (eds.): The Viruses, vol. 1, pp. 459–474. Academic Press, New York. 1959.
39. Foster, J. W.: Morphogenesis in bacteria: Some aspects of spore formation. Quart. Rev. Biol., 1956, **31**:102–118.
40. Gale, E. F.: Assimilation of amino acids by gram-positive bacteria and some actions of antibiotics thereon. Adv. Protein Chem., 1953, **8**:285–391.
41. Gale, E. F.: Synthesis and Organization in the Bacterial Cell. John Wiley and Sons, New York. 1959.
42. Gale, E. F., and Foulkes, J. P.: Effect of nucleic acid on protein synthesis and amino acid incorporation in disrupted staphylococcal cells. Nature, 1954, **173**:1223–1227.
43. Gale, E. F., and Foulkes, J. P.: The incorporation of labelled amino acids by disrupted staphylococcal cells. Biochem. J., 1955, **59**:661–675.
44. Gale, E. F., and Foulkes, J. P.: The effect of nucleic acids on the development of certain enzymic activities in disrupted staphylococcal cells. Biochem. J., 1955, **59**:675–684.
45. Gillies, R. R., and Duguid, J. P.: The fimbrial antigens of *Shigella flexneri*. J. Hyg., 1958, **56**:303–318.
46. Gottschalk, A.: Chemistry of virus receptors. In Burnet, F. M., and Stanley, W. M. (eds.): The Viruses, vol. 3, pp. 51–61. Academic Press, New York. 1959.
47. Grace, T. D. C.: Tissue culture for arthropod viruses. Trans. N. Y. Acad. Sci., 1959, Ser. II, **21**:237–241.

48. Gunsalus, I. C., and Stanier, R. Y. (eds.): The Bacteria, vol. 1: Structure. Academic Press, New York. 1960.
49. Halvorson, H. O. (ed.): Spores II. Burgess, Minneapolis. 1961.
50. Halvorson, H. O., and Church, B.: Biochemistry of spores of aerobic bacilli with special reference to germination. Bact. Rev., 1957, **21:**112–131.
51. Hartl, W.: Zum Pleomorphismus der Bakterien. Zent. f. Bakt., I. Abt., Orig., 1957, **167:**347–367.
52. d'Herelle, F.: Le Bacteriophage: son rôle dans l'immunité. Masson et Cie, Paris, 1921. English translation: Williams and Wilkins, Baltimore, 1922.
53. d'Herelle, F.: Le Bacteriophage et son comportment. Masson et Cie, 1926. English translation: Williams and Wilkins, Baltimore. 1926.
54. d'Herelle, F.: Bacteriophage and its Clinical Applications. Thomas, Springfield, Illinois. 1930.
55. Hess, E. L., and Lagg, S. E.: Some physical aspects of the bacterial cell. Science, 1958, **128:**356–358.
56. Higashi, N.: Electron microscopy of viruses in thin sections of cells grown in culture. Progress Med. Virol, 1959, **2:**43–72.
57. Hoffman, H., and Frank, M. E.: Form and internal structure of cellular aggregations in early *Escherichia coli* microcultures. J. Gen. Microbiol., 1961, **25:**353–364.
58. Holdsworth, E. S.: The nature of the cell wall of *Corynebacterium diphtheriae*. Isolation of an oligosaccharide. Biochim. Biophys. Acta, 1952, **9:**19–28.
59. Horne, R. W., Waterson, A. P., Wildy, P., and Farnham, A. E.: The structure and composition of the myxoviruses. I. Electron microscope studies of the structure of myxovirus particles by negative staining techniques. Virology, 1960, **11:**79–98.
60. Houwink, A. L.: A macromolecular monolayer in the cell wall of Spirillum spec. Biochim. Biophys. Acta, 1953. **10:**360–366.
61. Houwink, A. L., and van Iterson, W.: Electron microscopical observations on bacterial cytology. II. A study on flagellation. Biochim. Biophys. Acta, 1950, **5:**10–44.
62. Johnson, F. H., and Dennison, W. L.: The volume change accompanying the Quellung reaction of pneumococci. J. Immunol., 1944, **48:**317–323.
63. Klug, A., and Caspar, D. L. D.: The structure of small viruses. Adv. Virus Res., 1960, **7:** 225–325.
64. Knaysi, G.: Cytology of bacteria. Ann. Rev. Microbiol., 1956, **10:**253–274.
65. Knaysi, G.: Elements of Bacterial Cytology. Comstock, Ithaca, N. Y. 2nd ed., 1951.
66. Knight, C. A.: The chemical constitution of viruses. Adv. Virus Res., 1954, **2:**153–182.
67. Labaw, L. W., and Mosley, V. M.: Periodic structure in the flagella and cell walls of a bacterium. Biochim. Biophys. Acta, 1954, **15:**325–331.
68. Labaw, L. W., and Wyckoff, R. W. G.: The electron microscopy of tobacco necrosis virus crystals. J. Ultrastructure Res., 1958, **2:**8–15.
69. Lanni, F.: Immunogenetic dissection of the T-5 bacteriophage tail. Science, 1958, **128:** 839–840.
70. Leifson, E.: Atlas of Bacterial Flagellation. Academic Press, New York. 1960.
71. Levinson, H. S., and Wrigley, A. S.: Spore germination and emergence of *Bacillus megaterium*. Science, 1960, **131:**1382.
72. Luria, S. E.: The bacterial protoplasm: composition and organization. In Gunsalus, I. C., and Stanier, R. Y., (eds.) vol. I, pp. 1–34. Academic Press, New York. 1960.
73. Maaløe, O., and Birch-Anderson, A.: On the organization of the nuclear material in *Salmonella typhimurium*. Society for General Microbiology, Symposium No. 6, pp. 261–270. Cambridge University Press, 1956.
74. Malmgren, B., and Heden, C.-B.: Studies of the nucleotide metabolism of bacteria. I. Ultraviolet microspectroscopy as an aid in the study of the nucleotide content of bacteria. Acta Path. Microbiol. Scand., 1947, **24:**417–436.
75. Maramorosch, K.: Multiplication of plant viruses in insect vectors. Adv. Virus Res., 1955, **3:**221–249.
76. Markham, R.: The biochemistry of plant viruses. In Burnet, F. M., and Stanley, W. M. (eds.): The Viruses, vol. 2, pp. 33–126. Academic Press, New York. 1959.
77. Marr, A. G.: Enzyme localization in bacteria. Ann. Rev. Microbiol., 1960, **14:**241–260.
78. Marr, A. G.: Localization of enzymes in bacteria. In Gunsalus, I. C., and Stanier, R. Y. (eds.): The Bacteria, vol. I, pp. 443–468. Academic Press, New York. 1960.
79. Mattern, C. F. T., and DuBuy, H. G.: Purification and crystallization of Coxsackie virus. Science, 1956, **123:**1037–1038.
80. McCarty, McL.: The lysis of group A hemolytic streptococci by extracellular enzymes of *Streptomyces albus*. II. Nature of the cellular substrate attacked by the lytic enzyme. J. Exptl. Med., 1952, **96:**569–580.
81. McKinney, R. E.: Staining bacterial polysaccharides. J. Bact., 1953, **66:**453–454.
82. McQuillen, K.: Bacterial protoplasts. I. Protein and nucleic acid metabolism in protoplasts of *Bacillus megaterium*. Biochim. Biophys. Acta, 1955, **17:**382–390.
83. McQuillen, K.: Bacterial protoplasts. In Gunsalus, I. C., and Stanier, R. Y. (eds.): The Bacteria, vol. I, pp. 249–360. Academic Press, New York. 1960.
84. Menolasino, N. J., Goldin, M., and Hoffman, A.: Alteration in flagellar morphology induced by specific anti-flagellar serum. J. Bact., 1956, **71:**54–59.
85. Mickle, H.: Tissue disintegrator. J. Roy. Microscop. Soc., 1948, **68:**10–12.
86. Mitchell, P.: Transport of phosphate through an osmotic barrier. Society for Experimental Biology, Symposium No. 8, pp. 254–261. Academic Press, New York. 1954.
87. Mitchell, P.: Biochemical cytology of microorganisms. Ann. Rev. Microbiol., 1959, **13:** 407–440.
88. Mitchell, P., and Moyle, J.: The glycerophospho-protein complex envelope of *Micrococcus pyogenes*. J. Gen. Microbiol., 1951, **5:**981–992.
89. Mitchell, P., and Moyle, J.: Osmotic function and structure in bacteria. Society for General Microbiology, Symposium No. 6, pp. 150–180. Cambridge University Press, 1956.
90. Morgan, C., Rose, H. M., and Moore, D. H.:

Structure and development of viruses observed in the electron microscope. III. Influenza virus. J. Exptl. Med., 1956, **104:**171–181.
91. Morowitz, H. J.: The energy requirements for bacterial motility. Science, 1954, **119:**286.
92. Murray, R. G. E.: The internal structure of the cell. In Gunsalus, I. C., and Stanier, R. Y. (eds.): The Bacteria, vol. I, pp. 35–96. Academic Press, New York. 1960.
93. Ogiuti, K.: Untersuchungen über die Geschwindigkeit der Eigenbewegung von Bakterien. Japanese J. Exptl. Med., 1936, **14:**19–28.
94. Pauker, K., Birch-Anderson, A., and von Magnus, P.: Studies on the structure of influenza virus. I. Components of infectious and incomplete particles. Virology, 1959, **8:**1–20.
95. Pijper, A.: Bacterial flagella and motility. Ergeb. Microbiol. Immunitäts. u. exptl. Therap., 1957, **30:**39–91.
96. Pirie, N. W.: The anatomy of tobacco mosaic virus. Adv. Virus Res., 1957, **4:**159–190.
97. Putnam, F. W.: Biochemistry of viruses. Ann. Rev. Biochem., 1956, **25:**147–176.
98. Raettig, H.: Bakteriophagie 1917–1956. Gustav Fischer, Stuttgart. 1958.
99. Richards, O. W.: Phase microscopy 1950–1954. Science, 1954, **120:**631–639.
100. Roberts, R. B., and Roberts, I. Z.: Potassium metabolism in *Escherichia coli.* Interrelationship of potassium and phosphorus metabolism. J. Cell. Comp. Physiol., 1950, **36:**15–39.
101. Robinow, C. F.: The chromatin bodies of bacteria. Bact. Rev., 1956, **20:**207–242.
102. Robinow, C. F.: Morphology of bacterial spores, their development and germination. In Gunsalus, I. C., and Stanier, R. Y. (eds.): The Bacteria, vol. I, pp. 207–248. Academic Press, New York. 1960.
103. Robinow, C. F., and Murray, R. G. E.: The differentiation of cell wall, cytoplasmic membrane and cytoplasm of gram-positive bacteria by selective staining. Exptl. Cell Res., 1953, **4:**390–406.
104. Roger, F., and Roger, A.: Une variante simple d'emploi du colorant de Wright utilisable avec les rickettsies et les virus de groupe Trachome-Psittacose-Lymphogranulomatose. Bull. Soc. Path. Exot., 1958, **51:**307–309.
105. Rowen, J. W., and Norman, A.: The size of deoxyribonucleic acid molecules in *E. coli.* Arch. Biochem. Biophys., 1954, **51:**524–528.
106. Salton, M. R. J.: The nature of the cell walls of some gram-positive and gram-negative bacteria. Biochim. Biophys. Acta, 1952, **9:** 334–335.
107. Salton, M. R. J.: The composition of the cell walls of some gram-positive and gram-negative bacteria. Biochim. Biophys. Acta, 1953, **10:**512–523.
108. Salton, M. R. J.: Isolation of Streptomyces spp. capable of decomposing preparations of cell walls from various microorganisms and a comparison of their lytic activities with those of certain actinomycetes and myxobacteria. J. Gen. Microbiol., 1955, **12:**25–30.
109. Salton, M. R. J.: Bacterial cell walls. Society for General Microbiology, Symposium No. 6, pp. 81–110. Cambridge University Press, 1956.
110. Salton, M. R. J.: Surface layers of the bacterial cell. In Gunsalus, I. C., and Stanier, R. Y. (eds.): The Bacteria, vol. I, pp. 97–152. Academic Press, New York. 1960.
111. Salton, M. R. J.: The anatomy of the bacterial cell. Bact. Rev., 1961, **25:**77–99.
112. Salton, M. R. J.: Microbial Cell Walls. John Wiley and Sons, New York. 1961.
113. Salton, M. R. J., and Williams, R. C.: Electron microscopy of the cell walls of *Bacillus megatherium* and *Rhodospirillum rubrum.* Biochim. Biophys. Acta, 1954, **14:**455–458.
114. Schachman, H. K., and Williams, R. C.: The physical properties of infective particles. In Burnet, F. M., and Stanley, W. M. (eds.): The Viruses, vol. 1, pp. 223–228. Academic Press, New York. 1959.
115. Schaechter, M., et al.: Morphological, chemical and serological studies on the cell walls of *Rickettsia mooseri.* J. Bact., 1957, **74:**822–829.
116. Schäfer, W.: The comparative chemistry of infective virus particles and of other virus-specific products: Animal viruses. In Burnet, F. M., and Stanley, W. M. (eds.): The Viruses, vol. 1, pp. 475–504. Academic Press, New York. 1959.
117. Schaffer, F. L., and Schwerdt, C. E.: Purification and properties of poliovirus. Adv. Virus Res., 1959, **6:**159–204.
118. Schmidt, C. F.: The resistance of bacterial spores with reference to spore generation and its inhibition. Ann. Rev. Microbiol., 1955, **9:**387–400.
119. Schramm, G.: Biochemistry of viruses. Ann. Rev. Biochem., 1958, **27:**101–136.
120. Simpson, C. F., and White, F. H.: Electron microscope studies and staining reactions of leptospires. J. Infect. Dis., 1961, **109:**243–250.
121. Smith, K. M.: Morphology and development of insect viruses. Adv. Virus Res., 1955, **3:**199–220.
122. Smith, K. M.: Insect viruses. In Burnet, F. M., and Stanley, W. M. (eds.): The Viruses, vol. 3, pp. 369–392. Academic Press, New York. 1959.
123. Stanley, W. M.: Virus composition and structure—25 years ago and now. Fed. Proc., 1956, **15:**812–818.
124. Steinhaus, E. A.: Insect Microbiology. Comstock, Ithaca, N. Y. 1946.
125. Steinhaus, E. A.: Nomenclature and classification of insect viruses. Bact. Rev., 1949, **13:** 203–223.
126. Steinhaus, E. A.: Microbial diseases of insects. Ann. Rev. Microbiol., 1957, **11:**165–182.
127. Stent, G. S. (ed.): Papers on Bacterial Viruses. Little, Brown, and Co., Boston. 1960.
128. Stocker, B. A. D.: Bacterial flagella: morphology, constitution and inheritance. Society for General Microbiology, Symposium No. 6, pp. 19–40. Cambridge University Press, 1956.
129. Stocker, B. A. D., and Campbell, J. C.: The effect of nonlethal deflagellation on bacterial motility and observations on flagellar regeneration. J. Gen. Microbiol., 1959, **20:**670–685.
130. Stoker, M. G. P., Smith, K. M., and Fiset, P.: Internal structure of *Rickettsia burnetii* as shown by electron microscopy of thin sections. J. Gen. Microbiol., 1956, **15:**632–635.
131. Symposium: The Nature of the Bacterial Surface. Society for General Microbiology. Thomas, Springfield, Illinois. 1949.
132. Symposium: The biology of bacterial spores. Bact. Rev., 1952, **16:**89–143.
133. Symposium: Bacterial Anatomy. Society for

General Microbiology, Symposium No. 6. Cambridge University Press, 1956.
134. Symposium: Recent advances in the biochemistry of viruses. Fed. Proc., 1961, **20:**632–664.
135. Thompson, D'Arcy: On Growth and Form. Cambridge University Press. 2nd ed., 1952. Abridged ed.: Bonner, J. T. (ed.): Cambridge University Press, 1961.
136. Thorne, C. B.: Capsule formation and glutamyl polypeptide synthesis by *Bacillus anthracis* and *Bacillus subtilis*. Society for General Microbiology, Symposium No. 6, pp. 68–80. Cambridge University Press, 1956.
137. Tomcsik, J.: Bacterial capsules and their relation to the cell wall. Society for General Microbiology, Symposium No. 6, pp. 41–67. Cambridge University Press, 1956.
138. Tomcsik, J., and Guex-Holzer, S.: Änderung der Struktur der Bakterienzelle in Verlauf der Lysozyme Einwirkung. Schweiz. Z. allgem. Pathol. u. Bakteriol., 1952, **15:**517–525.
139. Valentine, R. C., and Isaacs, A.: The structure of the Newcastle disease-mumps-influenza (Myxovirus) group. J. Gen. Microbiol., 1957, **16:**680–685.
140. Weibull, C.: Characterization of the protoplasmic constituents of *Bacillus megaterium*. J. Bact., 1953, **66:**696–702.
141. Weibull, C.: Bacterial protoplasts. Ann. Rev. Microbiol., 1958, **12:**1–26.
142. Weibull, C.: Movement. In Gunsalus, I. C., and Stanier, R. Y. (eds.): The Bacteria, vol. I, pp. 153–206. Academic Press, New York. 1960.
143. Williams, R. C.: The size and shape of viruses as determined by electron microscopy. Cold Spring Harbor Symp. Quant. Biol., 1953, **18:**185–195.
144. Winkler, A.: Die Bakterienzelle. Gustav Fischer, Stuttgart. 1956.
145. Woese, C. R., and Forro, J. R.: Correlations between ribonucleic acid and deoxyribonucleic acid metabolism during spore germination. J. Bact., 1960, **80:**811–817.
146. Work, E.: Biochemistry of the bacterial cell wall. Nature, 1957, **179:**841–847.
147. Work, E.: The mucopeptides of bacterial cell walls. A review. J. Gen. Microbiol., 1961, **25:**167–189.
148. Wyckoff, R. W. G.: Electron Microscopy. Interscience, New York. 1949.

Chapter 4

THE GROWTH OF MICROORGANISMS

The growth of microorganisms is essentially the specific, balanced synthesis of the components of protoplasm from the nutritive substances present in the immediate environment. In addition, the newly synthesized constituents must be assembled and appropriately packaged to yield replicates of the original unit. The specificity of the entire process is dependent upon the operation of directive mechanisms, or genetic controls, which have the unique property of self-replication so that they appear as intrinsic parts of the new units.

This complex process is obviously dependent upon, and affected by, the kinds and concentrations of nutrient substances present in available form and upon a continuous supply of energy required for the endothermic reactions of synthesis. The source of the latter is the coupled oxidation-reduction reactions of respiration, so that, in the broad sense, the nutritive environment also includes oxidizable substrates together with reducible substances functioning as immediate and/or ultimate hydrogen acceptors.

Microbial growth may be approached in a number of ways. The most obvious are the cytological approach, that of the growth of microorganisms as population, and that of the nature of the biochemical processes involved. Such separations are artificial in that they represent no more than relative emphasis on one or another view of the same process but are useful for purposes of discussion. The first two will be considered here, and the last elsewhere (Chap. 5).

THE GROWTH OF BACTERIA

By far the most common method of multiplication of bacteria is that of simple binary fission in which the cell apparently constricts and separates into two daughter cells. Fission occurs in a single plane, at a point indicated by the development of a thickened, equatorial band in the cell wall, at a right angle to the long axis of bacillary and spiral forms. It may occur in one, two, or three planes of the spherical forms, making possible the characteristic morphological groupings described earlier (Chap. 3). Bacilli and spirochetes show some elongation prior to fission. The cocci do not, as a rule, although there may be some increase in the diameter of the cell without significant alteration of its spherical form. The size a single cell must reach before fission is quite constant, although

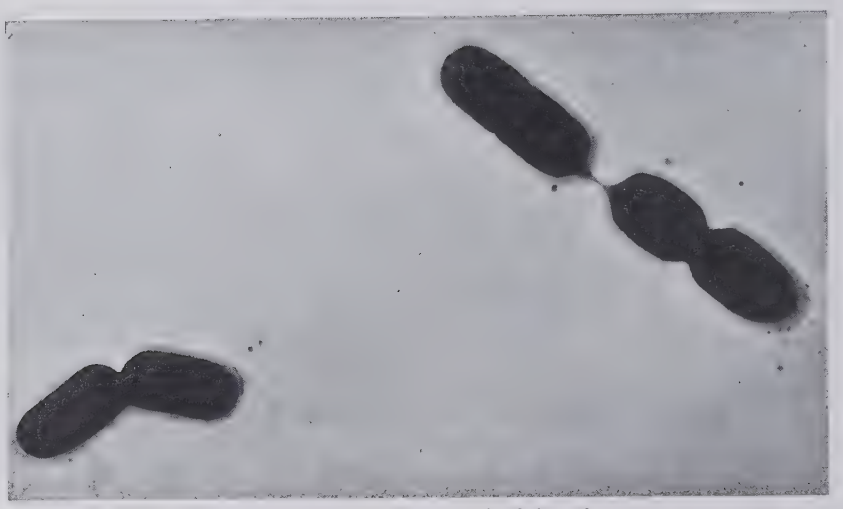


Figure 35. Electron micrograph of *Lactobacillus acidophilus* showing various stages in cell division. The initial slight constriction, deeper constriction, and partially separated daughter cells are all shown. Note the light cell walls surrounding the dense black protoplast, and the diploid cells connected by a delicate strand of protoplasm. (Mudd, Polevitsky, and Anderson.)

differences in this maximum are associated with the age of the culture (see below).

While cell division is clearly intimately related to the synthesis of cell substance, it may appear to be a distinct process in that, within limits, cell division may be inhibited by failure or inhibition of cell wall synthesis, while other components of the cell substance continue to be formed. One of the effects of penicillin, for example, is an inhibition of cell division of staphylococci, and the individual cells continue to grow without dividing and become greatly enlarged. Cell division may be inhibited in a number of other ways as, for example, by culture in magnesium (Mg^{++}) deficient mediums, by treatment with certain organic compounds such as 5-diazouracil, acriflavine, or methylenemelamine, or with ionizing radiation such as x-radiation or α-radiation. When rod-shaped bacteria are treated in this way, they fail to divide and continue to grow as long filaments. The nature of the processes interfered with is not known, but apparently phosphorus metabolism and the synthesis of DNA and RNA are not affected.[71, 133]

More precise definition of the internal structure of the bacterial cell has made more clear some cytological aspects of cell division. The first step is the formation of a septum, or cell plate, across or slightly oblique to the long axis in the case of bacillary forms, dividing the cell contents into approximately equal portions. This develops by centripetal growth of the plasma membrane, followed closely by the cell wall. The manner in which this occurs is not entirely clear, *viz.*, whether the plasma membrane grows by extension, or whether its formation is associated with the minute peripheral bodies that appear in electron micrographs of ultrathin sections to move inward with the centripetally growing cell wall.[20, 28] In any case, constriction of the cell occurs, and the two daughter cells separate. The septum may not be entirely closed when the cells separate, and they may remain connected for a short time by a thin strand of protoplasm.

While bacterial multiplication usually takes place by transverse fission, other kinds of cell division may occur. Some, such as the diphtheria and tubercle bacilli, show true branching that is characteristic of some of the higher fungi, and budding, similar to the budding of yeast cells, has been described by some workers. More complex processes occur, such as the formation within the cell of viable granules, or gonidia, which on liberation develop into morphologically typical

cells; this is firmly established as the so-called swarmer stage in the development of some soil bacteria, but is not otherwise of common occurrence. Another kind of more complex reproductive process occurs in the Bacteroides and pleuropneumonia-like organisms, or PPLO (Chap. 27), in which these structurally fragile cells swell to form large round bodies that then may undergo multipolar germination, with segmentation of outgrowing processes to form daughter cells.[115] All these more complex kinds of reproduction are quite uncommon in that they may occur in only a very few kinds of bacteria with some regularity, or extremely rarely in many kinds of bacteria.

The possibility of conjugation between unlike cells, and inferentially a sexual stage, in the reproduction of bacteria has been of perennial interest. Conjugation occurs between bacterial sexes (Chap. 7), and when the strains, differentiable with respect to biochemical markers such as nutritional requirements, are grown in mixed culture, hybrids appear in the proportions to be expected on the assumption of Mendelian segregation. While the occurrence of conjugation is firmly established, it is a rare event. Transfer of inheritable characteristics from one bacteria strain to another, however, may be accomplished in other ways also (Chap. 7), and the mixed character of progeny is not necessarily evidence of conjugation.

The generation time in binary fission, *i.e.*, the time elapsing between cell divisions, is minimal during the exponential growth phase (see below) of the bacterial culture. Certain enteric forms, such as the cholera vibrio and coliform bacilli, are among the most rapidly proliferating bacteria, with generation times of 20 minutes or less. More slowly growing bacteria, such as the tubercle bacilli, have generation times of several hours, or even days.[102]

The rapidity of cell division among the bacteria is not unique as sometimes supposed, for embryonic cells of higher forms may multiply equally rapidly. The unique aspect of the rate of bacterial multiplication lies, rather, in the fact that such a short time is required for the bacterial cell to reach full maturity.

The Growth of Bacterial Populations

For obvious practical reasons bacteria are ordinarily manipulated and studied, not as individuals, but as aggregates made up of very large numbers of cells. Bacterial growth may be considered as the growth of populations of many millions of cells whose characteristics are essentially statistical ones, and the behavior of individual cells is assayed as a frequency.

Growth of bacteria in culture may be considered from a number of points of view, and the methods of its measurement depend upon the purpose at hand. A simple view that is commonly, if tacitly, taken assumes that growth may be described by the reaction:

$$\text{culture medium} + O_2 \rightarrow \text{bacterial protoplasm} + \text{oxidized substance}$$

which proceeds from left to right, and eventually comes to an equilibrium. The reaction velocity constants may be determined by the rate of disappearance of the reacting substances or the rate of appearance of end products.

Although complicated by a variety of side reactions, the assumption of such a relation has been extremely useful in studies on bacterial metabolism. The growth of autotrophic bacteria, for example, is conveniently measured in terms of the conversion of carbonate to organic carbon. A somewhat more mechanistic concept is that of the instability of an inoculated culture medium which, after undergoing a series of complex changes, reaches a stable equilibrium at a lower energy level and provides the basis of studies on the energy metabolism of bacteria.

The most common method of measuring the growth of bacteria in culture is by periodic counting of the number of cells present. This may be a viable count, made by quantititative dilution and plate culture, or as total cells by direct cell count, or indirectly as turbidity trans-

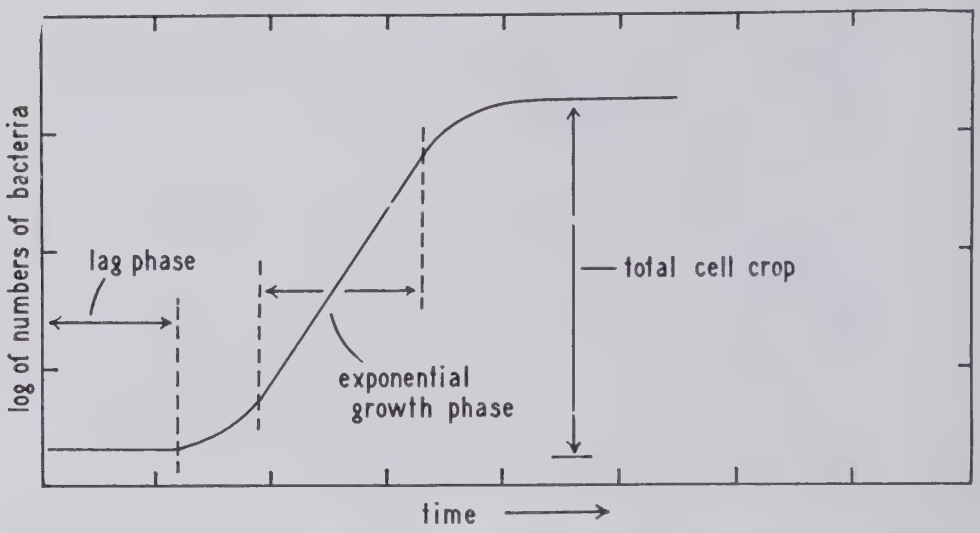


Figure 36. Diagrammatic representation of bacterial growth when the logarithms of the numbers of cells are plotted against incubation time.

lated into numbers with a previously prepared calibration curve. Or the total number of bacteria present may be measured as dry weight.

When all, or practically all, the cells are viable and multiplying by binary fission, the rate of growth is exponential. This potential rate of multiplication may be realized only up to a certain point. As numbers increase in the microcosm of the culture tube, competition between individual organisms for foodstuffs, oxygen, and the like, progressively reduces the opportunity for further growth until a saturating population density is reached.

If no increasingly effective retardation were operative, the potential increase would be expressed by the relation:

$$\frac{dY}{dt} = bY$$

where Y is equal to the number of individuals per unit volume, and b to the rate of growth, or generation time, of the microorganism. When there is a maximal possible cell density, K, this geometric rate of increase is only partially realized, the extent of the realization depending on how near the culture is to its maximum density at any given time. Or, mathematically:

$$\frac{dY}{dt} = bY\frac{K - Y}{K}$$

This is the differential equation of the logistic function:

$$Y = \frac{K}{1 + e^{a-bx}}$$

This function, plotting as a symmetrical S-shaped curve, describes the growth of populations of a variety of living organisms. If the numbers of bacteria in a growing culture are measured periodically and plotted against time of incubation, the points fall on a similar S-shaped curve, but one that is asymmetrical in that the point of inflection is not halfway between the upper and lower asymptotes but is considerably nearer the lower asymptote. Such curves are ordinarily plotted on semilog paper, *i.e.*, the logarithms of the numbers of bacteria are plotted against time on an arithmetic scale. This procedure gives a more nearly symmetrical curve and has the advantage, not only of minimizing errors in counting, but also, when microorganisms are multiplying at an exponential rate, of the points falling on a straight line, and the generation time being expressed as the slope of the straight-line portion of the curve.

The bacterial growth curve, shown in

schematic form in Figure 36, is divided into segments, or growth phases. The significant portions are the lag phase during which little or no increase in numbers occurs, the logarithmic or exponential growth rate phase during which the bacteria are multiplying at a maximum rate, and the stationary phase when the maximum total growth has been reached. The positive and negative growth acceleration phases at either end of the period of exponential growth are sometimes differentiated but are of limited utility. Following the stationary phase, death of some portion of the cells present usually occurs, rapidly or slowly and at an exponential rate; microbial death is considered elsewhere (Chap. 6).

It is obvious from even casual consideration of the development of the bacterial culture that descriptions such as "good growth" and "poor growth" have little meaning. Good growth may mean rapid growth and result from a relatively short latent or lag period and/or a short generation time during the exponential growth phase, or it may mean heavy growth in the sense that the maximal population density is high. These are not necessarily synonymous; the cholera vibrio, for example, grows equally rapidly in the logarithmic phase in aerated culture in peptone water, or peptone water enriched by the addition of serum to 10 percent, but the maximal density is considerably greater in the enriched medium. The meaning of "growth" is, then, variable, and the growth response, however and to what purpose it may be defined, is a function of the environment in the microcosm of the culture vessel.

The Lag Phase. When bacteria are inoculated into fresh medium an appreciable time, or latent period, is required for adaptation to an environment new in the sense that it differs from that of the established parent culture. There is convincing evidence[51] that this period is divisible into an apparent and a true lag phase. The first is a consequence of the presence of cells in the inoculum which are not viable in the sense that they will not reproduce, although they may continue to metabolize, and thus reduce the size of the effective inoculum. Within limits, the length of the lag phase is directly related to the size of the effective inoculum, and the numbers of cells present during the subsequent exponential growth phase at successive points in time may be predicted from these considerations.

The true lag phase, in contrast, is the period required for the viable cells of the inoculum to accumulate enzymes, diffusible coenzymes, and essential intermediates in the synthesis of cell substance to the balanced concentrations consistent with synthesis at a maximum rate. When the inoculum is taken from a culture in the exponential growth phase, the lag is largely, or entirely, eliminated since the cells are already in a state of physiological balance making possible synthesis at a maximal rate.

On the other hand, the lag phase may be prolonged indefinitely by interference with the processes of adaptation. Vigorous aeration, preventing the accumulation of small amounts of carbon dioxide from endogeneous respiration that are essential to growth, prevents growth and multiplication. Similarly, the addition of antibacterial substances whose activity is primarily bacteriostatic, *i.e.*, growth-inhibiting rather than killing, results in an indefinite extension of the lag phase. Such substances include those of practical importance as chemotherapeutic agents, such as the sulfonamides and certain of the antibiotics, as well as other substances such as certain dyes. These act by interfering with essential metabolic reactions, such as the synthesis of enzymes (Chap. 6), and when their effect is neutralized, as by *p*-aminobenzoic acid when growth is inhibited by sulfonamides, growth proceeds. The effect of many chemotherapeutic drugs is, then, in a sense an indefinite prolongation of the lag phase of bacterial growth.

The Exponential Growth Phase. The stage in the development of the bacterial culture during which the cells are replicating at a constant and geometric rate represents an approximate, limited, steady state in the relation between the

bacteria and their environment. The steady state is approximate because the environment is continuously altered by the microorganisms, and limited in that it persists over only a portion of the life history of the culture.

The rate of growth is obviously determined by limiting factors. These may be intrinsic in the structure of physiological potentiality of the cell, as, for example, the rates of diffusion across the osmotic barrier of the cell. Or they may be extrinsic in that they may be environmental factors, such as the concentration of an essential nutrient, concentration of oxidizable substrate, and the rate of diffusion of oxygen into the culture.

Continuous flow culture.[68] Bacterial growth in the logarithmic phase may be partially maintained by rapid successive transplant from this growth phase, but much more precisely by cultivation of the bacteria in a continuous flow of fresh medium. A considerable number of devices for this purpose have been constructed[96, 100] of which the chemostat and bactogen have been the most widely used. The former is operated by differential air pressure to provide a continuous supply of fresh medium to a small growth chamber as the used medium exhausted. In the latter the medium is continuously pumped into and out of a large rotating flask in which the bacteria grow in large volumes of culture medium. Both are operated at flow rates held at some fixed value below that required to maintain growth at a maximal rate. The steady state so obtained has been subjected to detailed mathematical analysis.[47, 91, 97, 101] A variation on this theme has been used in which the flow of fresh medium is controlled by a photoelectric system to maintain the numbers (turbidity) of bacteria at a constant level.[6, 16, 35]

Under such constant cultural conditions one factor in the environment, such as concentration of a nutrient substance, may be varied, while the others are held constant in excess so that rate-determining effects may be measured precisely. Further, the kinetics of formation of products of synthesis may be studied in relation to generation time which becomes an experimental parameter. Theoretically, such a culture could be maintained indefinitely, but in practice it is limited because of the instability of the directive mechanisms of synthesis, *i.e.*, mutations occur and alter the character of the microbial population. On the other hand, the factors affecting spontaneous mutation rates may be studied under constant defined environmental conditions; it has, for example, been possible to show that some compounds are antimutagenic in that they counteract the mutagenic activity of other compounds.[98]

Synchronous cell division.[111] In the usual bacterial culture cell division occurs at random to give, because of the large number of discrete events, a continuous increase in numbers of cells. By subjecting the culture to alternate incubation and chilling temperatures, it is possible to force the majority of the cells to divide within a short time and thus synchronize cell division. Studies on pneumococcus[58] and the mouse typhoid bacillus[78] have shown that the entire population is more susceptible to genetic transformation in the first instance, and to conversion to the lysogenic state (see below) in the second. Results such as these suggest that actively dividing microorganisms are relatively more susceptible to alteration of directive genetic mechanisms than are those in a resting state.

Cessation of Cell Division. Termination of the exponential phase of growth is marked by a relatively rapid decrease in the rate of cell division. Cell division does not stop entirely in the stationary phase, but the cells begin to die and occasional division counteracts deaths so that the number of viable microorganisms remains relatively constant for a time. This period may be relatively short so that within a few hours a decline in numbers of viable cells assumes an exponential rate, or the numbers of viable organisms may fluctuate about some median value that is usually less than the maximum cell density.

The decreasing rate of cell division may be accounted for in many ways, and different factors are operative with different microorganisms, and with the

same microorganism under different conditions. Depletion of essential food substances may be the limiting factor but is probably not important with most kinds of culture mediums. The metabolic rate may be reduced by a lessened availability of substrates for the respiratory processes; thus a carbohydrate substrate may be reduced in amount, or the rate of diffusion of oxygen may be insufficient at high cell densities. One of the most important kinds of factors is the accumulation of end products of metabolism, such as alcohols and organic acids from carbohydrates, to toxic concentrations. For example, the total cell crop obtainable from liquid culture is markedly increased by inclusion of a readily available source of energy, such as glucose, and vigorous aeration. The stirring effect of aeration keeps the substrate concentrations, including that of oxygen, more or less uniform throughout the medium and provides excess oxygen so that not only do the oxidations proceed more nearly to completion with reduced accumulation of organic acids and the like, but the energy yield is increased. Under conditions such as these, the limiting factor is glucose concentration, and a cycle of growth may often be started by the addition of more glucose and sufficient alkali to neutralize the small amounts of accumulated organic acids.

While these energy-yielding reactions and their products are an essential element in that they affect growth, their effect on growth is more precisely defined as that of control of biosynthesis,[37, 104] expressed as the induction of synthesis of new enzymes as in the early stages of growth, the inhibition of enzymatic activity, and the inhibition of enzyme synthesis. These matters are considered in detail elsewhere (Chap. 5).

Morphological Variation. Both the morphology and physiological activity of bacteria differ in the different growth phases of the culture. As growth gets under way, the maximum size that a given cell reaches before fission increases somewhat, and a large portion of the bacteria in a culture at this stage of development are appreciably larger than at other times. The cells stain evenly, and there is no evidence of granular structure even in those bacteria in which metachromatic granules are most readily demonstrable.

The rate of respiration per cell increases, reaching a maximum at the end of the phase of accelerating growth rate, and declining as the culture goes into the logarithmic growth phase. This increased metabolic rate is more apparent than real for it is quantitatively related to cell size.

The cell size decreases also as the culture goes into the logarithmic phase of growth, although the individual cells remain homogeneous and stain uniformly. By the time the stationary phase is reached, the bacterial cells are uniformly smaller, and in cultures of spore-forming bacteria, many are forming spores. When the viable count begins to decrease, the cells no longer stain uniformly, but begin to show granular structure, and involution forms appear.

This succession of morphological types, the large embryonic or young cells, the smaller, uniformly staining, mature forms of exponential growth, and the senescent degenerating forms appearing in older cultures, has been designated *cytomorphosis*[46] and is considered by some to be analogous to a similar succession of cell types in higher forms.

THE REPLICATION OF VIRUSES[17, 26, 121, 122, 124]

Since the viruses are obligate intracellular parasites, consisting essentially of little more than a directive mechanism, virus replication differs from bacterial multiplication in that it involves a specific deviation in host cell synthetic processes, and the finished product is not a complete physiological system. Virus synthesis can be studied against the base line of normal host cell metabolism in a number of ways, such as the persistence or alteration of metabolic reactions associated with the formation of virus.

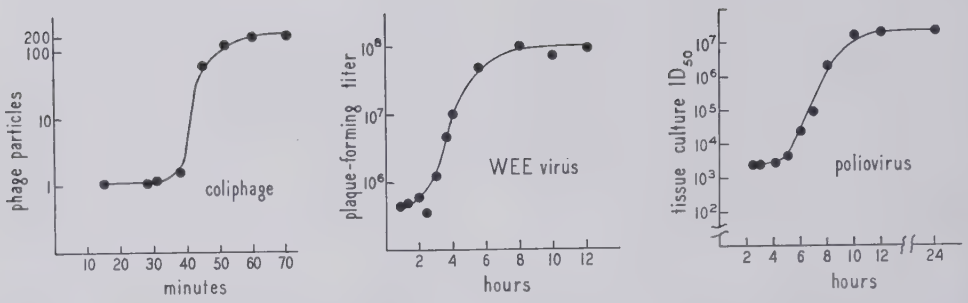


Figure 37. Growth curves of representative viruses. Coliphage and western equine encephalomyelitis virus are shown as one-step growth curves, the latter grown on chicken fibroblast culture and assayed by the plaque-counting method using a monolayer of chicken fibroblasts. The poliovirus type 3 was grown on HeLa cell culture and assayed by tissue culture ID_{50}. (Redrawn from Doermann, Dulbecco, and Ackermann and Francis respectively.)

Or, the relative proportions of original virus substance, host cell substance, and metabolic substrate present in the extracellular environment that make up the new virus substance may be determined.

The consequences of this parasitic relationship to the host cell are variable. In some instances virus proliferation results in destruction of the host cell, such as the lysis of the bacterial cell by bacteriophage, or the degenerative changes produced in host cells in tissue culture by many of the animal viruses. In other cases, the host cell is relatively unaffected in that it continues an apparently normal existence while supporting virus production, and in still others viral infection produces hyperplasia. These effects on the host cells in naturally occurring infections are in large part the basis of the pathogenesis and pathology of virus diseases.

Characteristics of Virus Replication. In general, a series of separable steps are involved in the proliferation of viruses. The first is an adsorption, more or less specific, of the infectious agent onto the surface of the host cell. Following adsorption, the particle penetrates the cell, and for an appreciable period, a few minutes to a few hours, cannot be detected as, for example, by breaking up the host cells and assaying the debris for virus activity. This is the latent, eclipse, or dark period during which the virus is said to be in the form of provirus. During this time the synthesis of virus substance is initiated, but active virus is not detectable until the infectious unit is organized through a process of maturation. Prior to this its presence is masked. Such an orderly sequence of events is inferred from the presence of "incomplete" virus in prematurely disrupted host cells, and its occurrence in mixture with complete virus under appropriate experimental conditions. There is no evidence suggesting that anything similar to binary fission of the intact virus particle occurs within the host cell.

In any case, when organization is complete, virus is liberated from the host cell at the end of the reproductive cycle, through breakdown of the cell structure or extrusion from the still functional cell. The liberation may be explosive, as with cell lysis, or virus may be liberated from the cell and shed into the environment over a period. Such a single cycle is called one-step growth. The free virus particles then invade other host cells and/or reinfect cells not destroyed in the previous process, and the cycle is repeated until the host cell substrate is exhausted. In the infected host organism virus replication may be inhibited by the development of an effective immune response and combination of the free virus particles with antibody to interfere with repetition of the reproductive cycle.

In one-step virus growth breakdown of host cells is only partially synchronized in the first cycle, and subsequently becomes random, while continuous liberation of virus from infected host

cells results in a continuous increase in virus. In either case, growth curves in which the amount, or titer, of virus is plotted against time are S-shaped and resemble the growth curves of bacterial cultures.

This general pattern of virus replication appears to hold for most of the viruses that have been sufficiently studied, with minor variations. Analogies may be drawn only with reservations, however, because it does not necessarily follow that similar stages in the development of widely different viruses are analogous.

The Replication of Bacteriophage[3, 12, 43, 49, 72, 79, 82, 85]

The bacterial viruses have been the most intensively studied of the viruses, largely for technical reasons, and are sometimes regarded as prototypes. While this may not hold true, the pattern of multiplication of these agents includes not only the stages observed with many other viruses, but also elaborations, some of which have not yet been observed with other viruses.

Adsorption.[69, 126, 134] The adsorption of the virus particle onto the surface of the bacterial cell is highly specific and determines in large part whether or not the bacterium is "susceptible" to infection or, conversely, whether the phage is able to infect the strain or species of bacterium. At least in the T-even coliphages, the portion of the differentiated phage particle structure concerned in adsorption is the terminal portion of the tail, and the phage is adsorbed tail-first on the bacterium. Adsorption is inhibited in the presence of antibody to the distal tail protein which is presumably covered with a layer of immune globulin, but antibody is not effective after adsorption has occurred.

The initial reaction consists of establishing electrostatic bonds between the phage tail and the bacterial cell surface. There is reason to believe that the bonding occurs between amino and carboxyl groups, the former predominating on the phage particle, and the latter on the bacterial cell wall.[103] There is some small but by no means complete correlation between the phage sensitivity of a bacterium and its somatic antigens. Phage does combine with purified polysaccharide-lipid somatic antigen, and with the lipocarbohydrate portion of the complex[70] of certain of the dysentery bacilli. The chemical substrate of the T-even coliphages is a similar substance, assaying 34 per cent lipid and 16.5 per cent reducing substances on hydrolysis,

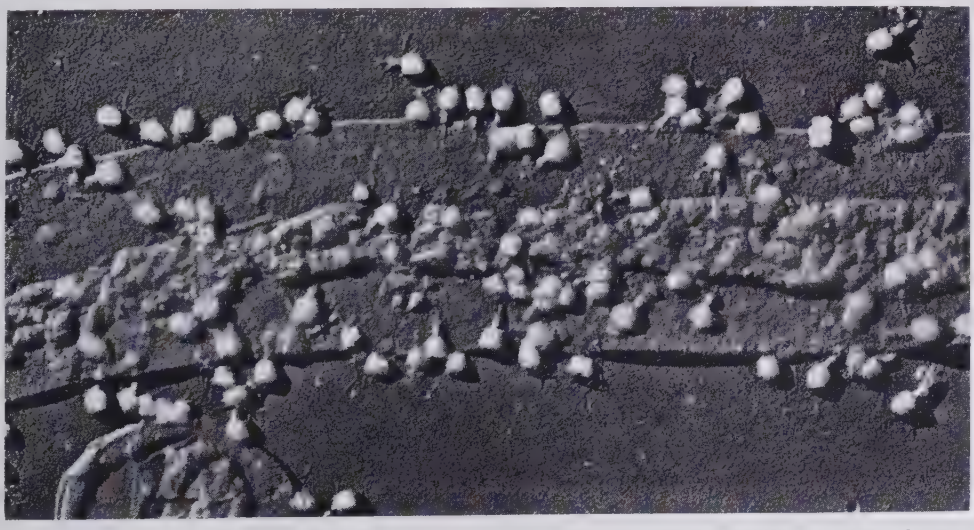


Figure 38. The interaction of T_2 coliphage wi h cell wall of *E. coli B*, showing the dissolution of the distal tail protein structure, in some cases leaving the protruding tail spike. The small rod-shaped particles may represent extruded spikes. × 30,000. (Kellenberger.)

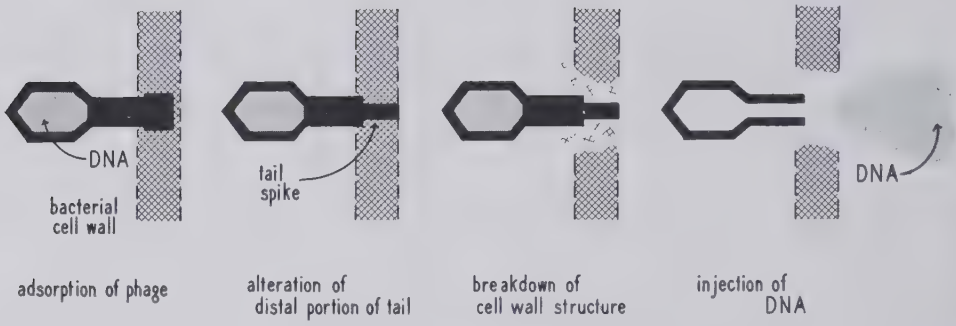


Figure 39. Diagrammatic representation of the sequence of events in the infection of the host cell by coliphage, proceeding from left to right. (Redrawn from Evans.)

and located in a spherical body deeply embedded in the bacterial cell wall.[135]

Infection of the Bacterium.[31, 32, 33] For a short time the adsorption of the phage particle on the bacterial cell wall is reversible, but within a few minutes it becomes irreversible when the distal tail protein breaks down, leaving the tail spike intact. As pointed out earlier (Chap. 3), the structure of the distal portion of the tail appears to depend upon the integrity of thiol ester linkages, and there is sufficient zinc in the bacterial cell wall to catalyze the dissolution of the structure.

Following this, the structure of the cell wall breaks down locally with liberation of soluble nitrogen compounds, and the osmotic barrier is affected, allowing some leakage of cell contents. This appears to be associated with contact between the cell wall and the exposed tail structure, resulting in the activation of a heat-labile enzyme which depolymerizes the cell wall structure.[15] Then the tail spike disappears, and the DNA content of the phage head structure is injected into the bacterial cell. The forces responsible for this are not known, but it is possibly relevant that, when the distal portion of the tail is removed by treatment with cadmium cyanide, emptying of DNA is accelerated in the presence of a variety of compounds, such as amino acids, amines, and amino sugars, having in common a positively charged amino nitrogen. On completion of this sequence of events, illustrated in schematic form in Figure 39, the attached phage particles may be broken off by treatment of the suspension in a blender. A short portion of the tail remains attached, and the remainder of the particle is an empty head and proximal tail membrane.

These events may also be produced *in vitro* in the sense of apart from the intact living bacterial cell. Partial dissolution of the distal tail structure has been referred to above. In addition, phage particles may be adsorbed on purified cell wall preparations or reacted with purified lipocarbohydrate substrate as just described. Soluble nitrogen compounds are liberated in the first instance, and, in both, phage DNA is discharged into the medium with consequent inactivation of the phage.[75, 76, 77]

Vegetative Virus.[73] The term vegatative virus has come to be used to designate proliferating intracellular virus in contrast to the resting stage status of extracellular, nonproliferating virus. The intracellular replication of bacteriophage occurs as a series of distinguishable morphological and biochemical[22] events.

Very soon after infection there is a disruption of the chromatinic body structure, and these bodies are no longer demonstrable as discrete entities. No other overt changes occur until near the completion of the growth cycle when the cell substance has become obviously altered to a filamentous, netlike reticular structure from whose interstices the mature phage particles have escaped.

When the processes of replication are periodically interrupted in various ways, such as mechanical or chemical (cya-

nide) disruption of the infected cell or inhibition of phage replication by treatment with substances such as proflavine or 5-methyl tryptophan, and the material examined for activity and morphological elements, an orderly process is suggested. During the early, or eclipse, portion of the latent period, neither phage activity nor distinctive morphological elements are demonstrable. Then spherical, empty bodies, containing little or no DNA, appear in increasing numbers, and these are regarded as phage particle precursors. By the middle of the latent period, mature infectious particles, complete with tails and DNA, may be found. These increase in number, presumably at the expense of the round particles since these decrease concurrently, until the full quota of infective particles, the so-called burst size, is reached, and the cell structure disintegrates to liberate them, leaving behind the reticulated structure described above. The mature phage particle, then, seems to be synthesized piecemeal and then assembled.

The only contribution made by the infecting particle to the new virus substance is a very small amount of DNA, less than 1 per cent of the total new viral DNA, and the remainder of the virus substance is synthesized by the host cell. Consistent with the morphological evidence, there is first an obligatory synthesis of viral protein very early in the latent period. DNA synthesis is occurring seven to eight minutes after the initiation of infection, with the first mature phage particles appearing 12 to 15 minutes from the beginning of the infection, and continues until all the phage particles are mature and the bacterial cell lyses.

The phage protein is derived in only minor part, 15 to 30 per cent, from the bacterial protein, and is synthesized largely from the constituents of the culture medium. In contrast, practically all of the bacterial DNA is broken down into small fragments and reassembled as phage DNA. When this is insufficient in amount, phage DNA is synthesized from the culture medium. In addition, in the case of the T-even coliphages, the DNA constituent 5-hydroxymethyl cytosine, which does not occur in bacterial DNA, is also synthesized, from bacterial cytosine or from the culture medium. The synthesis of this component would seem to necessitate the formation of enzymes not normally present in the bacterial cell.

Lysogeny.[10, 84] Two kinds of bacterial virus may be distinguished on the basis of their relation to the host bacterium. The one, virulent bacteriophage, produces the kind of infection of bacteria just described which culminates in lytic

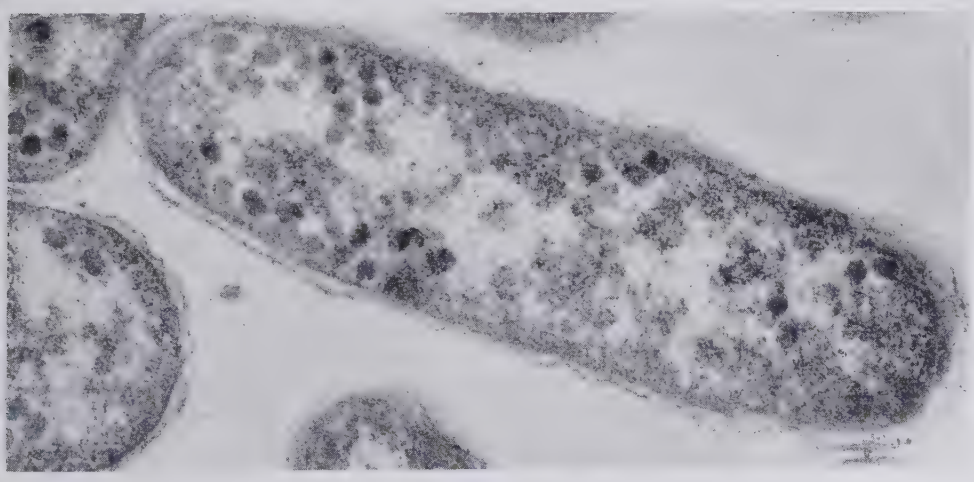


Figure 40. The intracellular growth of T_2 coliphage in *E. coli* B; note that only the polyhedral head portions of incomplete phage are present. Section fixed in osmium tetraoxide. × 60,000. (Kellenberger.)

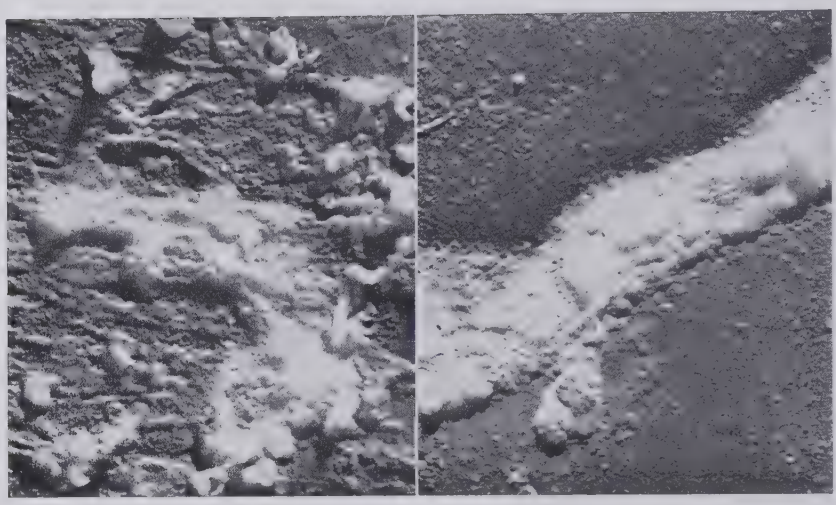


Figure 41. Electron micrographs of shadow-cast preparations of *E. coli* lysed by coliphage. The micrograph on the left shows the filamentous protoplasm; structure can be seen in the filaments just above the central mass, and the structure forms a net in the mass at the bottom. The separate opaque pieces casting long shadows are fragments of bacterial cell membrane. × 15,000. On the right the longitudinal filaments and interweaving cross-filaments are shown in an elongated bacillus undergoing lysis; note also the oblique net in the lower left corner. × 22,500. (Wyckoff.)

destruction of the host cell, and liberation of mature phage particles. The other kind, temperate bacteriophage, may give the production of active phage and its release by lysis, an abortive infection in which phage is not produced and disappears, or, finally, a nonlytic infection in which active phage is not produced but persists in an inactive form indefinitely, *i.e.*, becomes hereditary, in the bacterial host. Treatment of a susceptible strain of bacteria with a temperate phage usually gives some combination of these kinds of infection. A strain of bacteria infected in the last way is said to be lysogenic, and the phenomenon is called lysogeny. Lysogenicity tends to develop more frequently when there is multiple infection of the individual cell, *i.e.*, when the phage is present in excess.

Lysogeny may be mimicked by a kind of carrier state in which virulent phage coexists with phage-resistant bacteria, multiplying by infection and lysis of phage-sensitive mutants occurring occasionally in the bacterial population. The two kinds of association may be differentiated by culture of the bacteria in a medium containing antiphage serum. In the carrier state, or mixed culture, the phage occurs free and is neutralized by antibody so that it is unable to infect the occasional sensitive cell and disappears in serial culture. In the lysogenic strain, the phage persists within the bacterial cells and is transmitted from mother to daughter cell during cell division and so is not affected by serial culture in antibody-containing mediums.

The classic example of lysogenic bacteria is the strain of coliform bacilli, coli-Lisbonne, isolated in 1922 by Lisbonne and Carrere, which carries a Shiga dysentery bacillus phage. Many stock strains of bacteria are found, on examination, to be lysogenic, and possibly a majority carry phage.

The form in which the virus persists in the lysogenic bacterium is called provirus or prophage, but its nature is not clear. Like virulent phage in the eclipse stage of infection, it is not demonstrable in fragmented lysogenic bacteria, but there is reason to believe that the persistence of prophage represents more than an arrest of the normal processes of phage synthesis and replication. It is generally said that active phage is reduced to prophage, but it is not clear what is meant by reduction.

Virus may be shown to be present in the infected cells by treatment with

ultraviolet light, x-ray and other ionizing radiations, nitrogen mustard (methyl bis-[β-chloroethyl]-amine), peroxides, etc. Many of the bacteria are killed since the required dosage is high, but in those which survive, the processes of phage synthesis and assembly are induced. The bacteria are lysed within a short time, perhaps two hours, with liberation of active phage.

The interrelationships between temperate phages and their host bacteria are relatively complex. In general, a lysogenic bacterium cannot be lysed by the active form of the phage it is carrying, and is said to be "immune," a state reminiscent of immunity to superinfection familiar in diseases such as syphilis.[11, 136] Four other kinds of relationship are observed. First, there may be reciprocal cross-immunity between related phage strains. Second, there may be a reciprocal sensitivity, *i.e.*, the lysogenic bacterium carrying phage A may be superinfected with phage B, and vice versa. Third, there may be a partial dominance of one phage over another in that the lysogenic strain infected with phage A may be immune to phage B, but the lysogenic strain infected with phage B may be lysed by and/or superinfected with phage A. Finally, there may be complete dominance of one phage over another in that on superinfection the first phage is replaced by the second. In this last relation, the phage strains are said to be incompatible. Given the appropriate relation between phage strains, lysogeny may be multiple, and a lysogenic bacterial strain may carry several phages.

In bacteria infected with virulent phage, the entire directive mechanism of the host cell appears to be replaced by that of the virus, and the physiological system of the cell is literally under new management. In the lysogenic state the directive mechanism of the host cell is supplemented rather than replaced, and the host cell synthesizes provirus in addition to normal cell constituents.

The change may involve the synthesis of still other substances and, as well, minor modifications of the bacterial genetic apparatus. In consequence, characteristics of the bacterium, ordinarily regarded as inherent, biologically fundamental properties of the cell, may be attributable to, or modified by, the presence of phage in the lysogenic state.

These modifications are apparent in a number of ways. For example, the diphtheria bacillus is distinguished from the closely similar diphtheroid bacilli by its production of diphtheria toxin. Toxigenicity, however, has proved to be a characteristic only of lysogenic diphtheria bacilli, and may be removed by abolition of the lysogenic state or conferred on diphtheroid bacilli by making them lysogenic. Furthermore, characteristics of a lysogenic bacterium, either seemingly preexisting, such as biochemical characteristics or content of specific

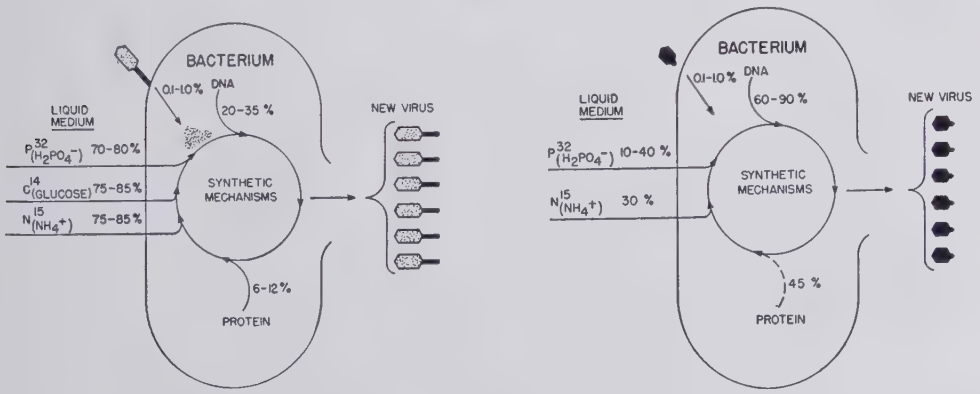


Figure 42. Diagrammatic representation of the source of phosphorus, carbon, and nitrogen in the synthesis of coliphage substance by the host cell. The percentages show the relative amounts contributed to the phage. *Left,* T-even coliphages; *right,* T_7 coliphage. (Evans.)

antigens, or acquired, such as drug resistance, may be transferred to another bacterium by making it lysogenic with phage derived from the first bacterium. This phenomenon of transduction, or infective heredity, is discussed elsewhere (Chap. 7). There seems to be, then, an extremely intimate relation between viral and bacterial genetic mechanisms in the lysogenic state.

The Replication of Animal Viruses[18, 50, 63, 83, 121]

Like the bacterial viruses, the animal viruses proliferate only on a living host cell substrate. Those suitable for this purpose are of two general kinds: the embryonated hen's egg and tissue cultures of animal cells such as epithelium and fibroblasts. Some kinds of cells may be maintained indefinitely in transplants like bacterial cultures, while others die off after a few transplants.

In either case, the growth substrate is actively metabolizing host cells which are induced to synthesize virus. The cycle of development is longer than that of the bacterial viruses; complete bacterial virus is formed as early as 20 minutes and seldom later than 90 minutes after the initiation of infection, while the growth cycle of animal viruses is a matter of hours, for example a minimum of four hours for influenza virus and seven hours for herpes simplex virus before the first complete virus is shed from the host cells.

While a living cell substrate is essential in the study of animal viruses, it is useful also in the study of pathogenic bacteria and host cell response. Meningitis may be produced in the chick embryo by the meningococcus; some of the early work with infectious agents in tissue culture concerned the growth of tubercle bacilli in macrophage cultures, and cultured tissue may produce antibody and show hypersensitivity reactions when the explants are taken from immunized animals.

Egg Culture. (Chap. 2.) The cells present in the embryonated egg, not only those of the embryo proper but also those of the chorioallantois, the membrane lining the allantoic cavity, and so forth, are susceptible to infection with agents to which the chicken is resistant. This is an expression of the general phenomenon of increase in resistance, or natural immunity, coincident with tissue differentiation. A useful variation is removal of the embryo and culture of viruses in the remaining cells, such as those lining the allantoic cavity, in the de-embryonated egg.

Viral growth may be supported by one tissue but not another, or at one stage of differentiation but not another. For example, rickettsiae are cultivable to large numbers in the yolk sac of the 6 to 8 day egg, equine encephalomyelitis virus in the tissues of 7 to 9 day embryos but in the yolk sac of the 15 day egg, influenza virus in the allantoic cavity of the 10 to 11 day egg, and vaccinia virus in the amnionic cavity of the 8 to 9 day egg when the rate of host cell multiplication is sufficient to replace those destroyed, and on the chorioallantois of the 12 to 14 day egg.

The pathology produced may simulate, to a greater or lesser degree, that of the infection in the natural host. The pox viruses, when cultured on the chorioallantoic membrane, produce pocks on the membrane with focal cell proliferation and subsequent necrosis and ulceration, and typical cytoplasmic inclusions. When the embryo is inoculated, tissues of endodermal and mesodermal origin are infected, and pock-like eruptions occur on the skin. Similarly, the virus of herpes simplex, when inoculated on the chorioallantois produces necrosis of ectodermal epithelium, and then infects mesodermal cells, and inclusions are found in endothelial cells. A herpetic encephalitis is produced in the embryo inoculated by the intracerebral route, and inoculation of the amnionic cavity results in vesicular lesions of the skin and mucous membranes of the pharynx in addition to a general infection of amnionic cells. On the other hand, cultivation of rickettsiae in the yolk sac, or influenza virus in the allantoic cavity,

does not result in pathology similar to the natural infection or that induced in experimental animals.

Tissue Culture.[107, 109, 120] (Chap. 2.) The culture of isolated tissues to provide a substrate for virus multiplication may be carried out in a number of ways, for example on a relatively large scale for the preparation of viral antigen and as many small individual cultures to allow titration of virus activity and/or its neutralization by antibody. Various kinds of tissue may be used, most commonly epithelium, macrophages, and fibroblasts, and one that has proved to be especially useful is the HeLa strain of malignant epithelial cells from a human cervical carcinoma.

Cytopathogenesis.[30] The proliferation of viruses is commonly, though not invariably, accompanied by changes in the host cells, the cytopathogenic effect of viruses. The kinds of visible changes produced allow the separation of viruses into groups, *viz.*, those that produce only cellular degeneration, those that produce degeneration and also form inclusion bodies in the degenerated cells, and those that produce multinucleated cells or multinucleated syncytial masses of protoplasm together with degenerative changes with or without the formation of inclusions.

The first group includes agents such as the polioviruses, the Coxsackie viruses, foot-and-mouth disease virus, and Newcastle disease virus (NDV); the second includes pox viruses such as mouse pox (ectromelia) and vaccinia, fowl plague virus, and the viruses of the psittacosis-lymphogranuloma group; and the third group includes viruses such as those of herpes simplex and herpes zoster and varicella. A fourth group of viruses which grow in tissue culture without apparent visible changes in the host cells includes the viruses of influenza, mumps, rabies, St. Louis encephalitis, and yellow fever.

Using the tissue culture technique and cytopathology as a criterion of viral activity, a considerable number of viruses have been isolated from normal as well as diseased individuals; their etiological relation to disease is, in many instances, as yet uncertain. These include viral agents from the respiratory tract, the adenoviruses or adeno-pharyngeal-conjunctival (APC) viruses; those isolated from the bowel, the enteric-cytopatho-

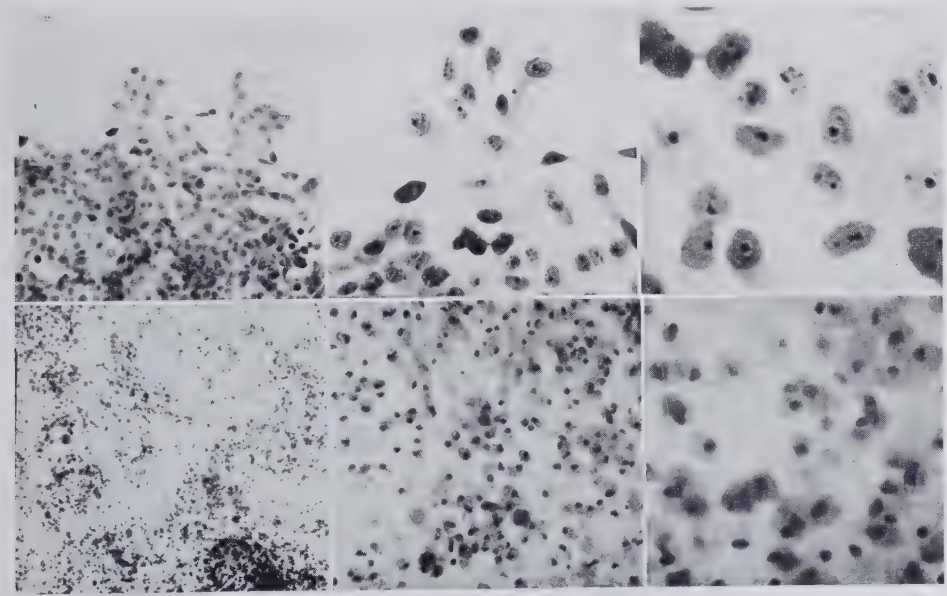


Figure 43. The cytopathology produced in HeLa cell culture of poliovirus type I. *Top row,* uninfected tissue shown at magnifications of × 65, × 160, and × 250, reading from left to right. *Bottom row,* degenerative changes produced in two days shown at the same magnifications reading from left to right. (Scherer, Syverton and Gey.)

genic-human-orphan (ECHO) viruses; and the agents isolated from normal monkey kidney tissue in connection with polio-virus culture in this tissue. Certain of the adenoviruses appear to be associated with upper respiratory disease and may be a factor in "virus pneumonia," and viruses of the ECHO group also appear to be related to disease.

Developmental Stages. The replication of animal viruses in egg or tissue culture follows a pattern similar to that described for bacterial virus, *i.e.*, adsorption onto the host cell, disappearance of viral activity during an eclipse period, and shedding of the mature virus. In contrast to the bacterial viruses which are released in "bursts" on lysis of the host cell, mature animal viruses are shed more or less continuously from the host cell.

Influenza virus.[45, 57] Proliferation of influenza virus in the entodermal cells lining the allantoic cavity of the embryonated hen's egg occurs readily, and it is one of the best known among the animal viruses in this respect. Following inoculation into the allantoic cavity of a 10 to 11 day embryo, the number of virus particles detectable in the allantoic fluid decreases at an exponential rate, about 50 per cent disappearing in a little over an hour, as the virus is adsorbed to the host cells. During this early stage, infection of the cells may be prevented by RDE, *i.e.*, neuraminidase, which destroys the red cell receptor functional in influenza virus hemagglutination (Chap. 3), and there is reason to believe that the host cell and red cell receptors are similar. Following adsorption, the virus particles enter the host cells, and the eclipse period lasts about four hours.

The first evidence of viral multiplication is the appearance in the middle of the eclipse period of soluble antigen, detectable by the complement-fixation test, which is not infective and has no hemagglutinin activity. In this connection it is of interest that, when the mature virus particle is disintegrated by shaking with ether, it breaks down to soluble complement-fixing antigen and hemagglutinin, suggesting that the soluble antigen may reflect an intermediate stage in virus synthesis.

Hemagglutinating activity, but not infectivity, is found in allantoic membrane extracts three to four hours after inoculation but does not increase in amount over that originally present in the inoculum until four hours and later. The hemagglutinating activity is not infective, and infective virus appears five to six hours after inoculation and is shed almost immediately into the allantoic fluid.

Morphological studies of ultrathin sections of allantoic membrane by electron microscopy at this time show both spherical mature virus particles and filamentous structures, but no characteristic changes in the nuclei or cytoplasm of the

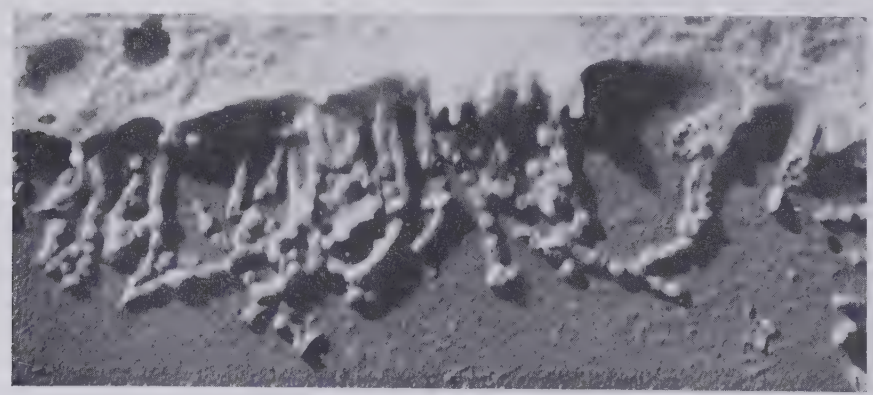


Figure 44. Shadow-cast ultrathin section of chorioallantoic membrane infected with influenza virus. The filaments extruded from the cells of the membrane appear to abstrict into spherical particles. × 10,000. (Wyckoff.)

host cell. The filaments appear to be formed by extrusion from the cell wall and are similar to those that may be produced by changes in osmotic balance. Both filaments and spheres appear to consist of a limiting membrane, a diffuse layer, and the spheres contain a dense central body about 20 mμ in diameter. The spheres seem to differentiate at or just beneath the cell surface, with assembly of the internal body, external layer, and limiting membrane.[93, 94]

The relation between the filaments and spheres is not entirely clear. It is believed by some that they are separate structures, but others believe that the filaments are, or differentiate into, rows of spheres. The filament is broken down by treatment with acid, and the spheres are digested by trypsin after acid treatment.[127]

However this may be, the spherical forms, 60 to 70 mμ in diameter, represent the mature, hemagglutinating, infectious form of the virus. About 40 to 60 such particles are liberated in one growth cycle. The cycle is repeated by infection and reinfection of the cells lining the allantoic cavity, with the accumulation of virus in the allantoic fluid, until the system is exhausted after about 48 hours.

Incomplete Virus.[34, 86, 112] Under appropriate circumstances incomplete, *i.e.*, functionally deficient in that it is noninfectious, influenza virus may be produced. Deficient virus particles occur when the allantoic cavity of the embryonated egg is inoculated with very large amounts of virus, when the virus is grown in the allantoic cavity of deembryonated eggs, or when nonneurotropic (nonadapted) strains of the virus are grown in the mouse brain.

In the first instance, the inoculum must be sufficiently heavy, more than 1 hemagglutinating unit (HU) of virus, to give multiple infections of the allantoic cells; there are about 1×10^8 cells lining the allantoic cavity, and 1 HU of virus contains 1×10^6 to 1×10^7 virus particles. Presumably the virus-synthesizing capacity of the allantoic cell is limited, and when this limit is exceeded the cell responds by incomplete synthesis of a portion of the virus formed. In the second instance, removal of the embryo seems to affect the allantoic cells in such a way that they can support only partial synthesis of virus. In the third, virus has not been adapted to the host cell substrate present in the central nervous system in the sense that these cells are not fully competent to synthesize it.

Incomplete or deficient virus differs from mature virus in that, while it agglutinates red blood cells and is specifically adsorbed to them, it is not only not infective and therefore unable to proliferate, but also it is smaller, with a sedimentation constant of 500 S as compared with one of 750 S for mature complete virus. A variety of considerations make it clear that incomplete virus is not a degradation product, but its appearance represents an arrest of the processes of synthesis and assembly of the mature virus particle.

Herpes simplex virus.[95, 106, 117, 118, 119, 130, 131] Studies on the growth of other animal viruses have shown that the same general sequence of events, *viz.*, adsorption, eclipse, and assembly and shedding of the virus, occurs with minor variations. Herpes simplex virus is of interest because of the formation of excess DNA within the nuclei of infected cells and the intranuclear formation of the virus. The virus is adsorbed rapidly on the chorioallantoic membrane of the embryonated egg, 50 to 80 per cent of the virus particles being adsorbed on the ectodermal cells in 15 minutes, and the adsorption is substantially complete in an hour. Adsorption is slower, *e.g.*, 50 per cent in one hour, and two hours are required for complete adsorption in HeLa cell culture. The latent period is 6 to 12 hours, and virus increases exponentially for 10 to 20 hours thereafter, but excess DNA is demonstrable in the nuclei of infected cells as early as 6 hours after infection. The virus particles are demonstrable within the nuclei as nucleic acid enclosed in a single-layered protein coat. A second protein coat is acquired elsewhere, presumably in the

cytoplasm, prior to shedding of the virus.

Poliovirus.[27, 56, 59, 60, 61, 89] Poliovirus grown in monkey kidney and other cell cultures is completely adsorbed under optimal conditions in two hours, and the eclipse period persists for three to four hours. New virus begins to accumulate intracellularly, and it has been reported that protein subunits, which appear in the cytoplasm, begin to aggregate as early as three hours after infection, leading to subsequent appearance of the typical icosahedral virus particle. There is evidence that protein and RNA are synthesized independently, but not that RNA synthesis occurs in the nucleus. Recombination experiments have suggested that the protein aggregation about viral RNA tends to be indiscriminate, but in general the processes of synthesis of viral substance and maturation of the vegetative particle are poorly understood. Complete virus begins to be shed as early as five hours after infection, apparently relatively rapidly by the individual cell, but shedding is asynchronous and continues in the culture for 18 to 24 hours after infection. The infected cells show marked morphological changes in nucleus and cytoplasm, tending to disintegrate as the virus is shed, and the cell is killed in a single cycle of virus replication.

Pox viruses.[19, 21, 38, 90, 114] Viruses of the pox group have been studied in some detail in this connection, and the observed morphological types of vegetative virus suggest an orderly sequence in the forms occurring during the process of replication. Unlike influenza virus, which seems to be assembled in final form at or near the cell surface and shed almost immediately into the environment, at least some of the pox viruses are formed in masses of differentiated protoplasm well within the cell, and the cytoplasm of the host cell may be filled with mature virus particles in the later stages of infection. When the masses of differentiated protoplasm are dense, they constitute the typical inclusion body, and when they are less dense, *i.e.*, inclusion bodies do not occur, the differentiated protoplasm constitutes a matrix from which the virus particles develop. This process has been studied in detail by Gaylord and Melnick[38] by electron microscopy of ultrathin sections of infected tissue embedded in methylacrylate, using vaccinia, ectromelia (mouse pox) and molluscum contagiosum viruses. These viruses are

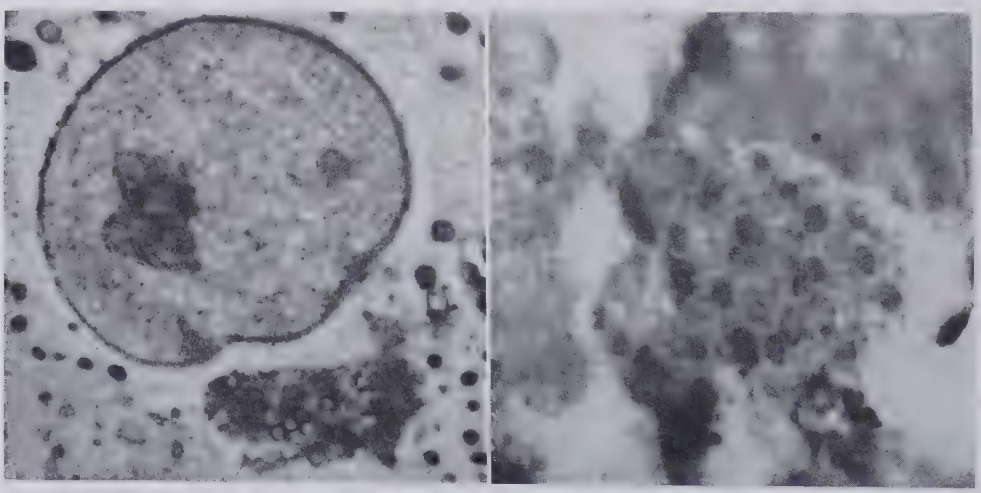


Figure 45. Electron micrographs of the developmental bodies of vaccinia virus. *Left*, early infection of a chorioallantoic cell of the embryonated egg, showing the nucleus of the cell and below it the matrix from which the virus particles originate. The developmental bodies in the matrix are few and appear to be "empty" membranes, and electron-dense mature virus particles are scattered in the cytoplasm. × 4000. *Right*, the differentiation of virus in an inclusion body in a rabbit corneal cell, showing the transformation of a part of the inclusion into virus particles, and developmental bodies of the virus in various stages of maturation. × 16,500. (Gaylord and Melnick.)

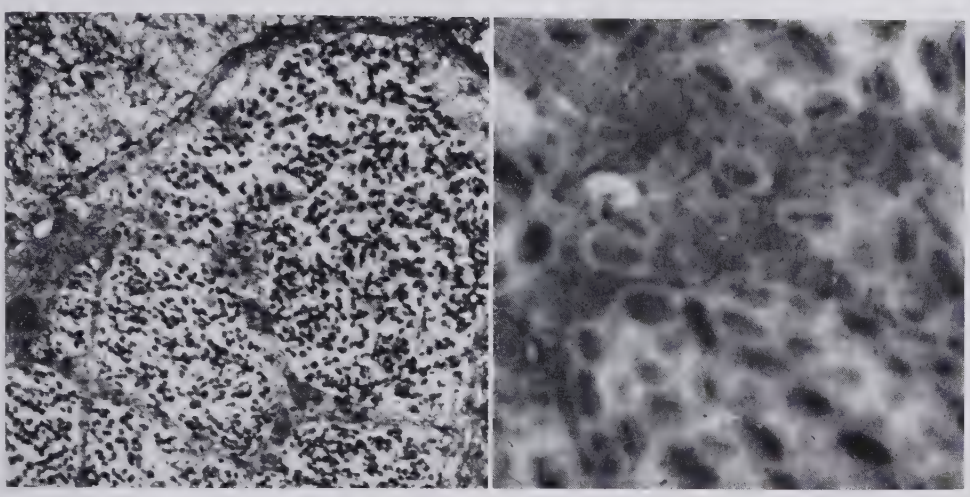


Figure 46. Electron micrographs of molluscum contagiosum virus in naturally infected cells. *Left,* a cell packed with mature virus particles separated into pockets by strands of cytoplasmic material. The nucleus is pushed to one side, and the nucleolus is very dense. × 8000. *Right,* virus particles under high magnification, showing all the developmental stages from the virus precursors clustered in the remnant of cytoplasm to those having bar- or dumbbell-shaped masses of dense material in the center, and the mature uniformly dense particles. × 26,000. (Gaylord and Melnick.)

similar with respect to the morphology of both developmental and mature forms.

During active multiplication several kinds of particles may be seen. These include hollow spheres, spheres filled with material of low electron density, spherical-to-ovoid forms in which there is a small round, or larger bar- or dumbbell-shaped body of high electron density, and finally ovoid bodies of uniform high electron density that represent mature virus. Since the hollow spheres predominate early in the infection, and those containing electron-dense material predominate late in the infection, it may be inferred that the several morphological types of particles arise from one another in orderly sequence, the hollow spheres representing an early stage in the developmental cycle. The process appears to be the same whether the virus is formed in an inclusion body or in a matrix, though possibly less confined in the latter instance. For example, the same developmental forms of vaccinia virus are found in the cells of the chorioallantoic membrane of the embryonated hen's eggs where inclusion bodies are not common and in the rabbit corneal cell where they are produced regularly.

Infectious Nucleic Acid.[24, 25, 48] It is apparent from the nature of the replication cycle of bacterial viruses that the entry of viral nucleic acid into the host cell is the essential feature which determines the synthesis of virus by the host. A corresponding function of RNA of many animal viruses is directly demonstrated by the preparation of RNA from the virus as infectious nucleic acid which will similarly infect the host cell and induce it to form, not only the nucleic acid, but also the complete vegetative virus particle. Extraction of RNA in 50 per cent phenol was described by Gierer and Schramm[39] for tobacco mosaic virus, and has subsequently been applied to a variety of animal viruses, for the most part small, neurotropic viruses, including poliovirus, the equine encephalomyelitis viruses, and certain of the arthropod-borne, ECHO, and Coxsackie viruses. The infectivity of such preparations is demonstrable by the inoculation of intact animals, the embryonated egg, and various kinds of tissue cultures, and is destroyed by treatment with ribonuclease. The yield of infectivity is low, commonly on the order of 0.1 per cent of the virus preparation from which the nucleic acid is extracted, but may be improved by use of hypertonic, on the order of 1 M., saline or sucrose solutions; such hyper-

tonic solutions apparently both stabilize the nucleic acid infectivity and facilitate its entrance into the host cells.

Certain observations with soluble preparations of DNA derived from bacterial virus may be analogous. Solutions of the virus substance obtained by osmotic shock lysis[116] and by extraction in concentrated urea[36] infect host cell protoplasts which are not infected by intact virus. The activity is presumably that of DNA, especially in the urea-treated preparations, but the presence of functional protein is not completely excluded.

Host Cells and Tissues.[1, 2, 7, 8, 23, 29, 40, 41, 74, 88, 99, 105, 123, 129] As pointed out earlier, the synthesis of virus by a host cell represents a deviation of the normal processes of metabolism through the imposition of a new directive mechanism. The characterization of the processes of virus synthesis against the base line of normal host synthesis is one of both theoretical and practical significance. For example, antibacterial substances having chemotherapeutic utility function, as noted elsewhere, by inhibition of growth through a differential effect on the microbial and host cells. The inefficacy of possible chemotherapeutic agents in the treatment of virus diseases, other than those of the psittacosis group, is taken to be a consequence in part of virus replication within the host cells, which afford some mechanical barrier to the agent, and, more significantly, of the utilization by the virus of host cell synthetic mechanisms. If the new sequence of metabolic events occurring in the infected cell can be sufficiently differentiated from normal cell metabolism, there is a possibility of selective interference with it.

In general, virus proliferation appears to be an obligate aerobic process, occurring most actively, and usually exclusively, in the actively metabolizing host cell. There is little overt evidence of infection, *viz.* the endogenous respiration rate is not altered by infection. When the tissue is glucose-starved, virus production is enhanced by the addition of glucose and, to a lesser extent, pyruvate or alanine. Intact functioning of the citric acid cycle is apparently essential, and virus production is inhibited by substances such as fluoroacetate and also by interference with the functioning of the hydrogen transport mechanisms by certain antibiotics (antimycin A). These are facets of the energy-producing metabolism of the host cell, located for the most part in the mitochondria, and the effects on virus production of interference with the system probably reflect no more than the requirement for adequate energy for virus synthesis.

Unfortunately so little is known of the normal synthetic processes of the host cell that it is not yet possible to equate these against the metabolism of the virus-infected cell. It is of interest that certain amino acid analogues, especially methoxinine, inhibit virus production, and that nucleic acid metabolism is accelerated in yolk sac culture of certain viruses of the psittacosis group with marked preferential incorporation of labeled phosphorus in the viral nucleic acids. In a general way, as illustrated in examples of both bacterial and animal virus growth described earlier, viral protein appears to be synthesized relatively early in the infection, and nucleic acid compounds a little later, but available information is as yet too meager to allow insight into the nature of the processes involved.

Cytotropism. There is a considerable degree of specificity between a virus and the kind of cell or tissue in which it is able to grow, *i.e.*, which provides an appropriate substrate. It has already been noted in connection with virus culture in the embryonated egg that some viruses will grow in some tissues but not in others. The combination of tissue differentiation in higher animals and the obligate intracellular growth of viruses provides the basis for tissue predilection or cytotropism of viruses. This is not a unique feature of these agents for pathogenic bacteria may also show a similar affinity for certain tissues such as the preferential infection of the central ner-

vous system by meningococcus, the predilection of typhoid and related bacilli for the bone marrow, and the relative noninfectivity of the cholera vibrio on parenteral inoculation, which is known as localization or elective localization rather than cytotropism.

Viruses are separable into a number of groups on this basis, that is, neurotropic, dermotropic, pneumotropic, and viscerotropic viruses. The neurotropic group includes agents such as the encephalitis viruses, which produce disease primarily of the central nervous system; the dermotropic group is exemplified by the pox viruses; the viscerotropic group is typified by yellow fever virus; and the pneumotropic group includes influenza, pneumonitis, and similar viruses.

Cytotropism is accentuated among the viruses, fundamentally because of their complete dependence upon host cells for proliferation, and superficially because the symptoms of the diseases they produce derive in large part from the pathology of some kinds of tissues to the relative exclusion of others. It is, however, regarded as of less importance than formerly because it may be altered, and because virus-infected tissues may show no pathology and not contribute to disease symptoms. In the first instance, for example, naturally occurring viscerotropic yellow fever virus, dermotropic vaccinia virus, and pneumotropic influenza virus may be made neurotropic by passage in the mouse brain, etc. In the second place, it has become increasingly clear that the pathogenesis of at least some virus diseases involves multiplication in tissues other than those associated with the symptoms of the disease; poliovirus, for example, apparently multiplies in the tissues of the alimentary tract without histopathology and reaches the central nervous system by hematogenous spread.

Cytotropism remains a fundamental characteristic of viruses in that it is a consequence of the ability of the virus to multiply more readily in one kind of cell than in another, but it is a characteristic that is subject to change.

Multiple Infections and Interference

In view of the obligate intracellular multiplication of viruses, the interesting question of whether or not multiple infections may occur presents itself. While the undoubtedly complex interrelationships of viruses with one another and with the host cell are not yet fully understood, it is clear that multiple, often double, infections may or may not occur, depending upon the viruses involved and upon the circumstances of infection.

Multiple Infections. Two kinds of multiple infections may occur. The one, multiple infection of an animal, is spurious for present purposes when different cells and tissues are involved. The other kind of multiple infection is that in which the same host cell is infected simultaneously with more than one kind of virus, supporting independent growth of each, and at the same time continuing, to some degree, its own metabolic activities. One such multiple infection, that of lysogenic bacteria with temperate bacterial viruses, has already been described. Among the other viruses, this may occur in some combinations of viruses, but not in others. One of the best examples is the simultaneous infection of the rabbit corneal cell with vaccinia and herpes simplex viruses in which the characteristic intranuclear herpetic inclusion and the cytoplasmic inclusion body of vaccinia may be found in the same cell. When the viruses are unrelated, such as these, the infections develop independently in that the viruses do not affect one another.

When a multiple infection with closely related viruses, such as the T-even coliphages or influenza virus variants, is produced by simultaneous exposure of the host cell, during the course of the mixed infection which occurs, there may be interaction between the two viruses, with recombination of characteristics and the appearance of hybrid progeny (Chap. 7).

Interference.[62, 80, 113, 128] Interference between viruses occurs when a second infection cannot be superimposed upon

an existing infection, or when one virus infects, *i.e.*, multiplies, to the exclusion of a second. In the first instance it is necessary to establish infection with the first virus by prior inoculation, allowing a period at least half as long as that required for a single growth cycle to elapse before infecting with the second. For example, while mixed infections with T-even coliphages may be produced by simultaneous inoculation as indicated above, if infection of the bacterium with one phage is first established, the cell cannot be infected with the other phage, and the same holds true for influenza virus strains inoculated into the allantoic cavity of the embryonated egg. Similarly, mumps and influenza viruses are incompatible in that infection with one cannot be superimposed on an existing infection with the other.

In the second instance, when the interfering virus is inoculated in very large amounts it markedly inhibits growth of the other virus, resulting in either apparently complete protection or an abortive infection. For example, the intracerebral inoculation of nonadapted influenza virus in mice, incapable of more than a single multiplication cycle in this tissue, gives a high degree of protection against virulent western equine encephalomyelitis virus by the same route for as long as a week, and normal susceptibility is not reached for three weeks. Similarly, under different experimental conditions, infection of rats with the same virus by the intranasal route is inhibited by St. Louis encephalitis or Japanese B encephalitis viruses given by the same route, with a reduction in mortality from 100 per cent to about 5 per cent. The latter kind of experiment illustrates the fact that interference is probably seldom fully effective, and some multiplication of the second virus may occur. It is not known whether this is due to a few host cells, which escaped the first inoculation, being infected by the second, or whether multiple infection occurs in some small number of cells.

Interference is not related to the initial adsorption stage of infection, nor is it produced by soluble components of viruses, but is apparently a consequence of the relation between the virus particle and the host cell after the synthetic mechanisms of the latter are affected.

While it is not considered to be interference, it may be noted that the initial adsorption may be prevented in a number of ways. The most obvious example is the prevention of influenza infection by destruction of the cell receptors for the virus with RDE, but this activity is ineffective after infection has occurred. When inoculated into the allantoic cavity of the embryonated egg after influenza virus infection has been initiated, RDE does not affect the first multiplication cycle, but prevents the occurrence of subsequent cycles. In view of the evidence suggesting that the receptor substance is mucoprotein in nature, it is of interest that similar substances of heterologous origin, such as certain tissue extracts or mucopolysaccharides of bacterial origin, will similarly prevent the initial adsorption. Presumably such substances compete with cell receptors for the virus. In fact, there are a number of antiviral "antibiotics" which prevent infection when administered prior to inoculation of virus.

Kinds of interference. The interference relationships between viruses are of two general kinds. The interference may be reciprocal in that either of a pair of viruses will prevent superinfection by the other. Or the interference may be effective in only one direction, *i.e.*, one virus may be unable to superinfect, but the other can infect in the presence of an infection with the former. A third kind of relationship is, of course, that of noninterference resulting in the development of multiple infections of the kind described earlier. There appears to be little correlation between the kinds of interference effects, or lack of them, and other characteristics of the viruses concerned, and in this sense interference is nonspecific.

Auto-interference. Attenuated or inactivated viruses may produce interference effects in heterologous systems, *i.e.*,

against diverse viruses, and they also inhibit multiplication of the corresponding homologous active virus. It was noted above that mixed infection of the allantoic cells of the embryonated egg may occur when the allantoic cavity is inoculated with variant strains of influenza virus. As in the case of the T-even coliphages, when infection with one strain is established, infection with the second does not occur. The "immunity" of lysogenic bacteria to lysis with the same phage in active form is possibly analogous, with provirus interfering with active virus synthesis. Protection of the fox against infection with virulent distemper virus by the inoculation, either simultaneously or during the early incubation period, with large amounts of the ferret-adapted virus is a similar interference phenomenon.

When inactivated virus is used to interfere, the way in which it is inactivated is of some importance. Inactivation of influenza virus by treatment with formaldehyde destroys the interfering property, inactivation by heat gives variable results, and inactivation by ultraviolet radiation gives uniformly effective interfering preparations. In the case of influenza virus, incomplete virus also interferes with subsequent infection with active complete virus.

Masked Virus. Auto-interference also occurs when mixtures of inactive and active virus are used to produce infection, provided that the former is present in great excess, *i.e.*, a thousandfold or more, and in sufficiently high concentration. Mixtures of active and inactive virus may be produced by actual mixing, by partial inactivation, intentional or unintentional, or when incomplete virus is formed in relatively large amount. When such a mixture is inoculated in cell culture or susceptible animals, there is little or no evidence of the presence of active virus because of the interfering effect of inactive virus, and the viral activity is said to be masked.

Such a masking effect may occur when seed virus is stored for long period or under conditions that cause the greater part of it to be inactivated. Similarly, this effect makes it difficult to titrate the end point of virus destruction with confidence, but in either case simple dilution of the inoculum may allow the demonstration of active virus if there is still sufficient present to allow the necessary dilution.

In animals infected with certain viruses, especially some tumor viruses, the activity may tend to disappear in serial animal passage by transplant of infected tissue, and the activity is said to be masked. It is possible that such masking may be due in part to the presence of inactive virus, but other factors, such as the concurrent presence of antibody, are significant also.

Nature of interference. It seems clear that viral interference needs to be considered as occurring at the cellular level. One of the more attractive theories of interference is that of a competitive inhibition of incompatible viral nucleic acids, but this is not completely consistent with the interfering effect of inactivated virus.

Interferon.[66, 67, 81] In some instances at least, interference is attributable to the activity of a substance, or group of substances, known as interferons, which are produced by host cell cultures in the presence of inactivated myxovirus, influenza virus for the most part. This substance, which is separable from the cell culture, appears to be a low molecular weight, basic, or histone-like, protein or polypeptide, and contains no nucleic acid. The substance is active against viruses other than the myxoviruses, including such apparently unrelated viruses as vaccinia, herpes simplex, and the equine encephalomyelitis viruses. Interferon produces an increased glycolysis in chick fibroblast cultures, and it has been postulated that it functions in inhibiting viral multiplication by uncoupling nuclear oxidative phosphorylation.[64, 65]

A similar substance, designated viral inhibiting fluid, or VIF, has been found to be produced in human kidney cell cultures of a chick embryo–adapted strain of type 2 MEF poliovirus. It too is apparently protein in nature, is inactivated

by trypsin but not by ribonuclease, and differs superficially from myxovirus interferon in that it is produced by the interaction of living, rather than inactivated, virus and host cells. VIF inhibits infection with other viruses such as Sindbis, vaccinia, and herpes simplex as well as other types of poliovirus.[54, 55] A closely similar inhibitor, which has been found to inhibit the infectivity of poliovirus RNA, has been obtained from Sindbis virus cultures.[53]

THE COMPARATIVE PHYSIOLOGY OF GROWTH

The physiological capabilities of microorganisms are an inverse reflection of their growth requirements in that the synthesis of cell substance from inorganic and simple organic compounds is a more complex process than that from more direct precursors of the constituents of protoplasm. Taken as a whole, microorganisms range in continuous series from the autotrophic forms capable of living in an inorganic environment, deriving energy from the oxidation of inorganic substrates or by a photosynthetic process, and assimilating nitrogen, carbon, etc., from inorganic sources, to the obligate intracellular parasites incapable of any, or at best rudimentary, independent physiological activity. The details of the processes of respiration, synthesis, and assimilation are considered elsewhere (Chap. 5).

In the strict sense of the term, only autotrophic microorganisms are not parasitic in that they are not dependent to some degree on other living organisms. Physiological limitations begin to appear in the heterotrophic forms, whose characteristic requirements of organic substrates for growth appear first as a requirement for organic carbon both as a source of energy derived by oxidation and as a source of intermediates for the synthesis of cell substance. Among the more fastidious kinds of microorganisms, the requirements for growth become more specific in that certain molecular structures, such as those represented in amino acids and fragments of enzymes or enzyme precursors, must be supplied preformed, but the microorganism is still physiologically independent in that it constitutes a complete physiological system.

Finally, the obligate intracellular parasites are set apart in that they are not complete physiological systems but are dependent upon the synthetic mechanisms of living host cells for their replication. It is more than coincidence that lack of independent metabolic activity is associated with the most minute forms, for there is literally not room within the small virus particle for the unknown but relatively large number of enzyme molecules required by a physiologically independent unit. The possible phylogenetic implications of the occurrence, or persistence, of the variety of physiological types of microorganisms are considered elsewhere (Chap. 7).

Since growth is the replication of the individual unit, and the replication is necessarily a directed process if individuality is maintained, microorganisms of diverse physiological capabilities may be equated on this common ground. The directive mechanism, or genetic control of synthesis, lies in pentose nucleic acid compounds, the deoxy form, or DNA, in bacteria and some viruses, and the RNA type in other viruses. The former is the substance of the chromosomes in the morphologically differentiated nucleus of the conventional cell, and the latter corresponds to the cytoplasmic gene substance.

The DNA molecule[132] is an asymmetrical, relatively rigid structure, occurring in the form of fibers about 2 $m\mu$ thick and several hundred $m\mu$ long. The structure is that of two chains of alternating purine and pyrimidine bases coiled in helical form around a common axis and joined by hydrogen bonds between the nucleotide bases. While the relation of this structure to that of the basic proteins, protamines and histones, that are combined with DNA in the living organism is not known, the regularity of the nucleic acid structure is consistent with the assumption that it may be the

molecular equivalent of the conventional chromosome and replicate in an analogous way, the helical structure providing a template for specific synthesis. The manner in which such a structure may exert a highly specific effect on cell metabolism is unknown. Too, there is considerable evidence[108] that biological information is stored and transmitted outside the DNA, *i.e.*, by nongenetic information mechanisms (plasmagenes, etc.) in the complete, functionally independent cell, but such nongenetic information is presumably lacking, or nearly so, in the smaller viruses, as indicated by the activity of infectious nucleic acid.

The growth of microorganisms can be regarded primarily as an expression of pentose nucleic acid replication, complicated to a greater or lesser extent by the side reactions of synthesis of the remainder of the unit. Such a view not only places the multiplication of diverse microorganisms on a common ground, but also provides a basic working hypothesis for an interpretation of the ways in which the hereditary mechanisms of these forms may be altered (Chap. 7).[122]

REFERENCES

1. Ackerman, W. W.: Mechanisms of persistent and masked infections in tissue culture. Ann. N. Y. Acad. Sci., 1957, **67**:392–402.
2. Ackerman, W. W., and Francis, T., Jr.: Characteristics of viral development in isolated animal tissues. Adv. Virus Res., 1954, **2**:81–108.
3. Adams, M. H.: Bacteriophages. Interscience, New York, 1959.
4. Afinsen, C. B.: Genetic control of protein structure in bacteriophage. Fed. Proc., 1961, **20**: 634–640.
5. Anderson, E. S., Armstrong, J. A., and Niven, J. S. F.: Fluorescent microscopy: Observation of virus growth with aminoacridines. Society for General Microbiology, Symposium No. 9, pp. 224–255. Cambridge University Press, 1959.
6. Anderson, P. A.: Automatic recording of growth rates of continuously cultured microorganisms. J. Gen. Physiol., 1953, **36**:733–737.
7. Bader, J. P., and Morgan, H. R.: Latent viral infection of cells in tissue culture. IX. Abortive infection with psittacosis virus. Proc. Soc. Exptl. Biol. Med., 1961, **106**:311–313.
8. Bauer, D. J.: Metabolic aspects of virus multiplication. Society for General Microbiology, Symposium No. 2, pp. 46–84. Cambridge University Press, 1953.
9. Bergmann, L.: Plant viruses in tissue culture. Trans. N. Y. Acad. Sci., 1959, Ser. II, **21**:227–236.
10. Bertani, G.: Lysogeny. Adv. Virus Res., 1958, **5**:151–193.
11. Boyd, J. S. K.: Immunity of lysogenic bacteria. Nature, 1956, **178**:141.
12. Boyd, J. S. K.: Bacteriophage. Biol. Rev., 1956, **31**:71–107.
13. Boyer, G. S., Denny, F. W., Jr., Miller, I., and Ginsberg, H. S.: Correlation of production of infectious virus with sequential stages of cytologic alteration in HeLa cells infected with adenovirus types 5 and 7. J. Exptl. Med., 1960, **112**:865–882.
14. Boyer, G. S., Leuchtenberger, C., and Ginsberg, H. S.: Cytological and cytochemical studies of HeLa cells infected with adenoviruses. J. Exptl. Med., 1957, **105**:195–216.
15. Brown, D. D., and Kozloff, L. M.: Morphological localization of the bacteriophage tail enzyme. J. Biol. Chem., 1957, **225**:1–11.
16. Bryson, V.: The turbidostatic selector—a device for automatic isolation of bacterial variants. Science, 1952, **116**:48–51.
17. Burnet, F. M.: Enzyme, Antigen and Virus. Cambridge University Press, 1956.
18. Burnet, F. M.: Principles of Animal Virology. Academic Press, New York. 2nd ed., 1960.
19. Cairns, J.: The initiation of vaccinia infections. Virology, 1960, **11**:603–623.
20. Chapman, G. B., and Hillier, J.: Electron microscopy of ultrathin sections of bacteria. I. Cellular division in *Bacillus cereus*. J. Bact., 1953, **66**:362–373.
21. Charles, A.: An electron microscope study of molluscum contagiosum. J. Hyg., 1960, **58**: 45–56.
22. Cohen, S. S.: Studies on controlling mechanisms in the metabolism of virus-infected bacteria. Cold Spring Harbor Symposia Quant. Biol., 1953, **18**:221–235.
23. Cohen, S. S.: Structural and chemical architecture of host cells with special reference to the synthesis of polymers. In Burnet, F. M., and Stanley, W. M. (eds.): The Viruses, vol. 1, pp. 15–222. Academic Press, New York. 1959.
24. Colter, J. S.: Nucleic acid as the carrier of viral activity. Progress Med. Virol., 1958, **1**:1–35.
25. Colter, J. S., and Ellem, K. A. O.: Interaction of viral nucleic acids with mammalian cells. Fed. Proc., 1961, **20**:650–655.
26. Cooper, P. D.: The chemical approach to the study of virus growth. Society for General Microbiology, Symposium No. 9, pp. 200–223. Cambridge University Press, 1959.
27. Darnell, J. E., Jr., and Eagle, H.: The biosynthesis of poliovirus in cell cultures. Adv. Virus Res., 1960, **7**:1–26.
28. Dawson, I. M., and Stern, H.: Structure in the bacterial cell wall during cell division. Biochim. Biophys. Acta, 1954, **13**:31–40.
29. Deinhardt, F., and Henle, G.: Studies on the viral spectra of tissue culture lines of human cells. J. Immunol., 1957, **79**:60–67.
30. Enders, J. F.: Cytopathology of virus infections (particular reference to tissue culture studies). Ann. Rev. Microbiol., 1954, **8**:473–502.
31. Evans, E. A., Jr.: Biochemical Studies of Bacterial Viruses. University of Chicago Press, 1952.
32. Evans, E. A., Jr.: Bacterial viruses (with partic-

ular reference to synthesis of). Ann. Rev. Microbiol., 1954, **8:**237–256.
33. Evans, E. A., Jr.: Bacteriophage as nucleoprotein. Fed. Proc., 1956, **15:**827–832.
34. Fazekas de St. Groth, S., and Graham, D. M.: The production of incomplete influenza virus particles among influenza strains: Chemical induction of the von Magnus phenomenon. Brit. J. Exptl. Path., 1955, **36:**205–213.
35. Fox, M. S., and Szilard, L.: A device for growing bacterial populations under steady-state conditions. J. Gen. Physiol., 1955, **39:**261–266.
36. Fraser, D., Mahler, H. R., Shug, A. L., and Thomas, C. A.: The infection of subcellular *Escherichia coli*, strain B, with a DNA preparation from T_2 bacteriophage. Proc. Nat. Acad. Sci., 1957, **43:**939–947.
37. Gale, E. F.: Synthesis and Organization in the Bacterial Cell. John Wiley and Sons, New York. 1959.
38. Gaylord, W. H., and Melnick, J. L.: Intracellular forms of pox viruses as shown by the electron microscope (vaccinia, ectromelia, molluscum contagiosum). J. Exptl. Med., 1953, **98:**157–172.
39. Gierer, A., and Schramm, G.: Infectivity of ribonucleic acid from tobacco mosaic virus. Nature, 1956, **177:**702–703. Die Infektiosität der Nucleinsäure aus Tabakmosaikvirus. Z. Naturforsch., 1956, **116:**138–142.
40. Ginsberg, H. S.: The significance of the viral carrier state in tissue culture systems. Progress Med. Virol., 1958, **1:**36–58.
41. Ginsberg, H. S.: Biological and biochemical basis for cell injury by animal viruses. Fed. Proc., 1961, **20:**656–660.
42. Godman, G. C., Morgan, C., Breitenfeld, P. M., and Rose, H. M.: A correlative study by electron and light microscopy of the development of type 5 adenovirus. II. Light microscopy. J. Exptl. Med., 1960, **112:**383–402.
43. Groman, N. B.: Phage-host relationships in some genera of medical significance. Ann. Rev. Microbiol., 1961, **15:**153–176.
44. Harrison, B. D.: The multiplication of viruses in plants. Society for General Microbiology, Symposium No. 9, pp. 34–60. Cambridge University Press, 1959.
45. Henle, W.: Multiplication of influenza virus in the entodermal cells of the allantois of the chick embryo. Adv. Virus Res., 1953, **1:**142–228.
46. Henrici, A. T.: Morphologic Variation and the Rate of Growth of Bacteria. Thomas, Springfield, Illinois. 1928.
47. Herbert, D., Elsworth, R., and Telling, R. C.: The continuous culture of bacteria: A theoretical and experimental study. J. Gen. Microbiol., 1956, **14:**601–622.
48. Herriott, R. M.: Infectious nucleic acids, a new dimension in virology. Science, 1961, **134:**256–260.
49. Hershey, A. D.: Bacteriophages as genetic and biochemical systems. Adv. Virus Res., 1957, **4:**25–61.
50. Higashi, N.: Electron microscopy of viruses in thin sections of cells grown in culture. Progress Med. Virol., 1959, **2:**43–72.
51. Hinshelwood, C. N.: Chemical Kinetics of the Bacterial Cell. Clarendon Press, Oxford. 1946.
52. Hirst, G. K.: Studies of mixed infections with NDV, poliovirus and influenza. Society for General Microbiology, Symposium No. 9, pp. 82–101. Cambridge University Press, 1959.
53. Ho, M.: Inhibition of the infectivity of poliovirus ribonucleic acid by an interferon. Proc. Soc. Exptl. Biol. Med., 1961, **107:**639–644.
54. Ho, M., and Enders, J. F.: An inhibitor of viral activity appearing in infected cell cultures. Proc. Nat. Acad. Sci., 1959, **45:**385–389.
55. Ho, M., and Enders, J. F.: Further studies on an inhibitor of viral activity appearing in infected cell cultures and its role in chronic viral infections. Virology, 1959, **9:**446–477.
56. Holland, J. J., and McLaren, L. C.: The mammalian cell-virus relationship. II. Adsorption, reception and eclipse of poliovirus by HeLa cells. J. Exptl. Med., 1959, **109:**487–504.
57. Horsfall, F. L.: Reproduction of influenza viruses. Quantitative investigations with particle enumeration procedures on the dynamics of influenza A and B virus reproduction. J. Exptl. Med., 1955, **102:**441–473.
58. Hotchkiss, R. D.: Cyclical behavior in pneumococcal growth and transformability occasioned by environmental changes. Proc. Nat. Acad. Sci., 1954, **40:**49–55.
59. Howes, D. W.: The growth cycle of poliovirus in cultured cells. III. The asynchronous response of HeLa cells multiply infected with type 1 poliovirus. Virology, 1959, **9:**110–126.
60. Howes, D. W.: The growth cycle of poliovirus in cultured cells. II. Maturation and release of virus in suspended cell populations. Virology, 1959, **9:**96–109.
61. Howes, D. W., and Melnick, J. L.: The growth cycle of poliovirus in monkey kidney cells. Virology, 1957, **4:**97–108.
62. Isaacs, A.: Viral interference. Society for General Microbiology, Symposium No. 9, pp. 102–121. Cambridge University Press, 1959.
63. Isaacs, A.: Biological aspects of intracellular stages of virus growth. In Burnet, F. M., and Stanley, W. M. (eds.): The Viruses, vol. 3, pp. 111–156. Academic Press, New York. 1959.
64. Isaacs, A.: Metabolic effects of interferon on chick fibroblasts. Virology, 1960, **10:**144–146.
65. Isaacs, A., Klemperer, H. G., and Hitchcock, G.: Studies on the mechanism of action of interferon. Virology, 1961, **13:**191–199.
66. Isaacs, A., and Lindenmann, J.: Virus interference. I. The interferon. Proc. Roy. Soc., Series B, 1957, **147:**258–267.
67. Isaacs, A., Lindenmann, J., and Valentine, R. C.: Virus interference. II. Some properties of interferon. Proc. Roy. Soc., Series B, 1957, **147:**268–273.
68. James, T. W.: Continuous culture of microorganisms. Ann. Rev. Microbiol., 1961, **15:**27–46.
69. Jesaitis, M. A., and Goebel, W. F.: The interaction between T-4 phage and the specific lipocarbohydrate of phase II *Shigella sonnei*. Cold Spring Harbor Symposia Quant. Biol., 1953, **18:**205–208.
70. Jesaitis, M. A., and Goebel, W. F.: Lysis of T-4 by the specific lipocarbohydrate of phase II *Shigella sonnei*. J. Exptl. Med., 1955, **102:**733–752.
71. Katchman, B. J., Spoerl, E., and Smith, H. E.: Effects of cell division inhibition on phosphorus metabolism of *Escherichia coli*. Science, 1955, **121:**97–98.

72. Kellenberger, E.: Growth of bacteriophage. Society for General Microbiology, Symposium No. 9, pp. 11–33. Cambridge University Press, 1959.
73. Kellenberger, E.: Vegetative bacteriophage and the maturation of the virus particle. Adv. Virus Res., 1961, **8:**1–61.
74. Koppelman, R., and Evans, E. A., Jr.: The metabolism of virus-infected animal cells. Progress Med. Virol., 1959, **2:**73–105.
75. Kozloff, L. M., and Lute, M.: Viral invasion. II. The role of zinc in bacteriophage invasion. J. Biol. Chem., 1957, **228:**529–536.
76. Kozloff, L. M., and Lute, M.: Viral invasion. III. The release of viral nucleic acid from its protein covering. J. Biol. Chem., 1957, **228:** 537–546.
77. Kozloff, L. M., Lute, M., and Henderson, K.: Viral invasion. I. Rupture of thiol ester bonds in the bacteriophage tail. J. Biol. Chem., 1957, **228:**511–528.
78. Lark, K. G., and Maaløe, O.: The induction of cellular and nuclear division in *Salmonella typhimurium* by temperature shifts. Biochim. Biophys. Acta, 1954, **15:**345–356.
79. Latarjet, R.: The multiplication of bacterial viruses studied by radiobiological methods. Society for General Microbiology, Symposium No. 2, pp. 175–193. Cambridge University Press, 1953.
80. Lindenmann, J.: Neuere Aspekte der Virus-Interferenz. Ergbn. Mikrobiol. Immunitätsf., 1960, **33:**369–397.
81. Lindenmann, J., Burke, D. C., and Isaacs, A.: Studies on the production, mode of action and properties of interferon. Brit. J. Exptl. Path., 1957, **38:**551–562.
82. Luria, S. W.: An analysis of bacteriophage multiplication. Society for General Microbiology, Symposium No. 2, pp. 99–118. Cambridge University Press, 1953.
83. Luria, S. W.: The reproduction of viruses: A comparative survey. In Burnet, F. M., and Stanley, W. M. (eds.): The Viruses, vol. 1, pp. 549–568. Academic Press, New York. 1959.
84. Lwoff, A.: Lysogeny. Bact. Rev., 1953, **17:**269–337.
85. Lwoff, A.: The nature of phage reproduction. Society for General Microbiology, Symposium No. 2, pp. 149–174. Cambridge University Press, 1953.
86. von Magnus, P.: Incomplete forms of influenza virus. Adv. Virus Res., 1954, **2:**59–80.
87. Markham, R.: Virus nucleic acids. Adv. Virus Res., 1953, **1:**315–332.
88. McCloskey, R. V., and Morgan, H. R.: Latent viral infection of cells in tissue culture. VIII. Morphological observations of psittacosis virus in L cell. Proc. Soc. Exptl. Biol. Med., 1961, **106:**85–88.
89. McLaren, L. C., Holland, J. J., and Syverton, J. T.: The mammalian cell-virus relationship. I. Attachment of poliovirus to cultivated cells of primate and nonprimate origin. J. Exptl. Med., 1959, **109:**475–485.
90. Melnick, J. L., et al.: Electron microscopy of viruses of human papilloma, molluscum contagiosum, and vaccinia, including observations of the formation of virus within the cell. Ann. N. Y. Acad. Sci., 1952, **54:**1214–1225.
91. Monod, J.: La technique de culture continué. Théorie et application. Ann. Inst. Pasteur, 1950, **79:**390–409.
92. Morgan, C., Godman, G. C., Breitenfeld, P. M., and Rose, H. M.: A correlative study by electron and light microscopy of the development of type 5 adenovirus. I. Electron microscopy. J. Exptl. Med., 1960, **112:**373–382.
93. Morgan, C., and Rose, H. M.: Electron-microscope observations on adenovirus and viruses of the influenza group. Society for General Microbiology, Symposium No. 9, pp. 256–272. Cambridge University Press, 1959.
94. Morgan, C., Rose, H. M., and Moore, D. H.: Structure and development of viruses observed in the electron microscope. III. Influenza virus. J. Exptl. Med., 1956, **104:**171–181.
95. Morgan, C., Rose, H. M., Holden, M., and Jones, E. P.: Electron microscope observations on the development of herpes simplex virus. J. Exptl. Med., 1959, **110:**643–656.
96. Novick, A.: Growth of bacteria. Ann. Rev. Microbiol., 1955, **9:**97–110.
97. Novick, A., and Szilard, L.: Description of the chemostat. Science, 1950, **112:**715–716.
98. Novick, A., and Szilard, L.: Anti-mutagens. Nature, 1952, **170:**926–927.
99. Pearson, H. E.: Biochemical aspects of viral growth. Ann. Rev. Microbiol., 1953, **7:**179–196.
100. Perret, C. J.: An apparatus for the continuous culture of bacteria at constant population density. J. Gen. Microbiol., 1957, **16:**250–264.
101. Powell, E. O.: Growth rate and generation time of bacteria, with special reference to continuous culture. J. Gen. Microbiol., 1956, **15:**492–511.
102. Powell, E. O.: An outline of the pattern of bacterial generation times. J. Gen. Microbiol., 1958, **18:**382–417.
103. Puck, T. T., and Tolmach, L. J.: The mechanism of virus attachment to host cells. IV. Physicochemical studies on virus and cell surface groups. Arch. Biochem. Biophys., 1954, **51:**229–245.
104. Roberts, R. B., McQuillen, K., and Roberts, I. Z.: Biosynthetic aspects of metabolism. Ann. Rev. Microbiol., 1959, **13:**1–48.
105. Rose, H. M., and Morgan, C.: Fine structure of virus-infected cells. Ann. Rev. Microbiol., 1960, **14:**217–240.
106. Ross, R., and Orlans, E.: The redistribution of nucleic acid and the appearance of specific antigen in HeLa cells infected with herpes virus. J. Path. Bact., 1958, **76:**393–402.
107. Ross, J. D., and Syverton, J. T.: Use of tissue cultures in virus research. Ann. Rev. Microbiol., 1957, **11:**459–508.
108. Salser, W.: Non-genetic information mechanisms. Perspect. Biol. Med., 1961, **4:**177–198.
109. Salzman, N. P.: Animal cell cultures. Science, 1961, **133:**1559–1565.
110. Schäfer, W.: Some observations concerning the reproduction of RNA-containing animal viruses. Society for General Microbiology, Symposium No. 9, pp. 61–81. Cambridge University Press, 1959.
111. Scherbaum, O. H.: Synchronous division of microorganisms. Ann. Rev. Microbiol., 1950, **14:**283–310.
112. Schlesinger, R. W.: The relation of functionally deficient forms of influenza virus to viral development. Cold Spring Harbor Symposia Quant. Biol., 1953, **18:**55–60.

113. Schlesinger, R. W.: Interference between animal viruses. In Burnet, F. M., and Stanley, W. M. (eds.): The Viruses, vol. 3, pp. 157–194. Academic Press, New York. 1959.
114. Smith, K. O., and Sharp, D. G.: Interaction of virus with cells in tissue cultures. I. Adsorption on and growths of vaccinia virus in L cells. Virology, 1960, **11:**519–532.
115. Smith, W. E., Mudd, S., and Hillier, J.: L-type variation and bacterial reproduction by large bodies as seen in electron micrograph studies of *Bacteroides funduliformis*. J. Bact., 1948, **56:**603–618.
116. Spizizen, J.: Infection of protoplasts by disrupted T-2 virus. Proc. Nat. Acad. Sci., 1957, **43:**694–701.
117. Stoker, M. G. P.: Mode of intercellular transfer of herpes virus. Nature, 1958, **182:**1525–1526.
118. Stoker, M. G. P.: Growth studies with herpes virus. Society for General Microbiology, Symposium No. 9, pp. 142–170. Cambridge University Press, 1959.
119. Stoker, M. G. P., Smith, K. M., and Ross, R. W.: Electron microscope studies of HeLa cells infected with herpes virus. J. Gen. Microbiol., 1958, **19:**244–249.
120. Swim, H. E.: Microbiological aspects of tissue culture. Ann. Rev. Microbiol., 1959, **13:**141–176.
121. Symposium: The Nature of Virus Multiplication. Society for General Microbiology, Symposium No. 2. Cambridge University Press, 1953.
122. Symposium: The Biological Replication of Macromolecules. Society for Experimental Biology, Symposium No. 12. Academic Press, New York. 1958.
123. Symposium: The cytopathology of virus infection. Ann. N. Y. Acad. Sci., 1959, **81:**1–214.
124. Symposium: Virus Growth and Variation. Society for General Microbiology, Symposium No. 9. Cambridge University Press, 1959.
125. Symposium: Recent advances in the biochemistry of viruses. Fed. Proc., 1961, **20:**632–664.
126. Tolmach, L. J.: Attachment and penetration of cells by viruses. Adv. Virus Res., 1957, **4:** 63–110.
127. Valentine, R. C., and Isaacs, A.: The structure of influenza virus filaments and spheres. J. Gen. Microbiol., 1957, **16:**195–204.
128. Wagner, R. R.: Viral interference. Some considerations of basic mechanisms and their potential relationship to host resistance. Bact. Rev., 1960, **24:**151–176.
129. Walker, D. L.: In vitro cell-virus relationships resulting in cell death. Ann. Rev. Microbiol., 1960, **14:**177–196.
130. Watkins, J. F.: The adsorption, penetration and eclipse of herpes simplex virus in chorioallantoic membrane ectoderm. J. Gen. Microbiol., 1960, **22:**40–56.
131. Watkins, J. F.: The early stages of infection of HeLa cells with herpes simplex virus. J. Gen. Microbiol., 1960, **22:**57–71.
132. Watson, J. D., and Crick, F. H. C.: The structure of DNA. Cold Spring Harbor Symposia Quant. Biol., 1953, **18:**123–131.
133. Webb, M.: Effects of magnesium on cellular division in bacteria. Science, 1953, **118:**607–611.
134. Weidel, W.: Bacterial viruses (with particular reference to adsorption/penetration). Ann. Rev. Microbiol., 1958, **12:**27–48.
135. Weidel, W., and Kellenberger, E.: The *E. coli* B receptor for phage T-5. II. Electron microscopic studies. Biochim. Biophys. Acta, 1955, **17:**1–9.
136. Zinder, N. D.: Lysogenization and superinfection immunity in Salmonella. Virology, 1958, **5:**291–326.

Chapter 5

BACTERIAL METABOLISM

by James W. Moulder, Ph.D.

Bacterial physiology, or bacterial metabolism, deals with the biochemical activities of living bacteria. Since bacteria are small, unicellular organisms, their physiology is a cellular physiology and is best treated in biochemical terms. The biochemical changes brought about through the activities of bacteria are by far the most obvious manifestations of their existence. The long-known but little-understood phenomena of fermentation, putrefaction and decay, soil fertility, and infectious diseases of man and lower animals are each one of the many facets of the biochemical potentiality of these microorganisms. The oxidation of ammonia by the nitrifying bacteria, the decomposition of carbohydrates to alcohols and acids, and the production of substances toxic to higher animals are a part of the normal activities of this heterogeneous group of living organisms. The elucidation of many of these phenomena has not only made possible a partial control of a considerable portion of man's environment but has also provided an insight into the life processes of bacteria, organisms structurally so simple that morphology cannot carry us far in their study.

Morphologically, bacteria are simple organisms and are often regarded as a primitive type of cell. However, these organisms are not simple biochemically. Many of them oxidize glucose, for example, by mechanisms very similar to and often more complex than those used by higher animals. Others, such as the nitrifying bacteria, whose respiratory processes consist of but a single simple oxidation, synthesize all the components of protoplasm from inorganic compounds of carbon and nitrogen—a more complex process than that of joining together preformed amino acids. Since bacteria metabolize in these and other highly complex ways, it is difficult to regard them as physiologically simple. There is, however, some reason to think that bacteria are primitive cells. The multiplicity of reactions which bacteria are able to bring about suggests that, in these organisms, the original capabilities of protoplasm have not been lost to the extent that they have been by the differentiated cells of higher organisms. The animal body, for example, has no mechanism for the breakdown of cellulose, yet bacteria with the ability to hydrolyze this substance are universally distributed in the soil. Bacteria capable of breaking down a particular organic compound have been isolated since the early days of bacteriology by the relatively simple expedient of inoculating a highly specific medium containing the substance under consideration as the only source of energy with a complex mixture of orga-

nisms such as exist in samples of soil. It is probable that by this method a bacterium could be found which would oxidize almost any organic compound. No group of higher organisms shows such a high degree of physiological flexibility. The wide range of carbon compounds that a single bacterial species may oxidize, and the multiplicity of end products of the fermentation of a single carbohydrate, likewise point toward such a flexibility. The simplicity and variability of the nutritive requirements of many bacteria also suggest a physiologically primitive cell.

The physiological economy of the cell is dynamic rather than static, and life is compatible only with a state of greater or lesser, but nevertheless continuous, physiological activity. This activity may be approximately separated into two phases: the oxidative, energy-yielding reactions of respiration, and the energy-requiring reactions of synthesis. The former make possible a continuous, regulated supply of energy to the cell, while the latter channel this energy into the synthesis of cell substance. The separation of the processes of respiration and synthesis cannot be complete, of course, for in many instances the products of the respiratory process are assimilated by the cell and utilized for synthetic purposes; in fact in many instances a preliminary decomposition is necessary before assimilation can occur.

One of the fundamental tenets of modern physiology and biochemistry is the belief that the chemical composition of the protoplasm of all organisms is essentially the same and that it is synthesized by the same kind of chemical reactions; *i.e.*, the metabolism of all living things fits a single general pattern, an undoubted reflection of a common evolutionary origin. Protoplasm is considered to be made up of a large number of essential metabolites, substances which are essential for the metabolism and life of all cells. These essential metabolites are synthesized by the same mechanisms and enter into the same reactions in organisms of widely diverse phylogenetic origin. This concept of the unity of biochemical processes does not mean, of course, that the composition and metabolism of all cells is exactly the same. Some substances are found only in a single organism, or in a highly restricted group of organisms, while the same substance may be synthesized or broken down by entirely different mechanisms in different species. Indeed, these differences are often of great importance, such as those which make possible the successful chemotherapy of bacterial infections. However, the concept is a generally useful one.

RESPIRATION

In its broadest sense, bacterial respiration refers to all the energy-yielding reactions occurring within the cell. Except in photosynthetic organisms, these reactions consist of the oxidation of chemical substances present in the medium in which the bacteria are growing. Respiration has two main and often inseparable functions: (1) it furnishes the energy needed for the life and growth of bacteria and (2) it transforms a considerable portion of the compounds oxidized as energy sources into substances which are assimilated and synthesized into new cell material.

SOME BASIC CONCEPTS OF RESPIRATION

When one substance is oxidized, another must simultaneously be reduced. The oxidized substance loses electrons which are gained by the one reduced. This transfer of electrons is easily seen in the oxidation-reduction of inorganic ions. In the example given here, Fe^{+++} clearly accepts an electron from Cu^{+} and is thus reduced to Fe^{++}.

$$Cu^{+} + Fe^{+++} \rightleftharpoons Cu^{++} + Fe^{++}$$

When organic compounds are oxidized, as in most reactions of bacterial respiration, hydrogen atoms are transferred as well as electrons. Thus, malic acid transfers two hydrogen atoms and two electrons to pyruvic acid with the result that malic acid is oxidized and pyruvic acid is reduced, as shown in **2** below.

The majority of biological oxidations may be most simply considered in terms of *hydrogen transfer,* although it must be remembered that electrons are transferred in the oxidation of organic compounds, just as in the oxidation of inorganic ions.

Using the concept of hydrogen transfer, any biological oxidation may be considered to be a special case of this general reaction.

$$A{-}H_2 + B \rightleftharpoons A + B{-}H_2$$

$A{-}H_2$ is the substance oxidized or the *hydrogen donor,* while B is the substance reduced or the *hydrogen acceptor.* One other component of the system is still needed. The oxidation-reductions of respiration do not proceed spontaneously, but require the presence of specific cell catalysts, the *respiratory enzymes.*

Biological oxidations are usually classified as either *aerobic* or *anaerobic.* If the hydrogen acceptor is molecular oxygen, *i.e.,*

$$A{-}H_2 + \tfrac{1}{2}\,O_2 \rightarrow A + H_2O$$

the oxidation is said to be aerobic. If a substance other than oxygen serves as the hydrogen acceptor, the reaction is anaerobic. Anaerobic oxidation-reductions are often referred to as *fermentations.* Although the presence or absence of oxygen is of critical importance in the metabolism of many bacteria, the basic mechanisms of aerobic and anaerobic respiration are essentially the same.

Energy Sources for Bacteria. Bacteria make use of a far wider variety of energy sources than any other group of organisms. Photosynthetic bacteria utilize the energy of visible light, chemoautotrophic organisms oxidize inorganic compounds as their sole source of energy, and heterotrophic bacteria oxidize almost every known type of organic compound. The table below (**1**) gives a few representative examples. In each case, the indicated reaction furnishes all the energy needed for growth of that species. The carbohydrates are the most widely utilized energy sources for he-

1.

Some Typical Energy-Yielding Reactions in Bacteria

ORGANISM	REACTION
Nitrosomonas europaea	$NH_3 + 1\tfrac{1}{2}\,O_2 \rightarrow HNO_2 + H_2O$
Thiobacillus thiooxidans	$S + 1\tfrac{1}{2}\,O_2 + H_2O \rightarrow H_2SO_4$
Thiobacillus denitrificans	$5\,S + 6\,KNO_3 + 2\,H_2O \rightarrow K_2SO_4 + 4\,KHSO_4 + 3\,N_2$
Hydrogenomonas pantotropha	$H_2 + \tfrac{1}{2}\,O_2 \rightarrow H_2O$
Methanobacterium omelianskii	$4\,H_2 + CO_2 \rightarrow CH_4 + 2\,H_2O$
Escherichia coli	$\underset{\textit{glucose}}{C_6H_{12}O_6} + 6\,O_2 \rightarrow 6\,CO_2 + 6\,H_2O$
Lactobacillus casei	$\underset{\textit{glucose}}{C_6H_{12}O_6} \rightarrow 2\,\underset{\textit{lactic acid}}{CH_3CHOHCOOH}$
Clostridium kluyveri	$\underset{\textit{acetic acid}}{CH_3COOH} + \underset{\textit{ethanol}}{CH_3CH_2OH} \rightarrow \underset{\textit{butyric acid}}{CH_3CH_2CH_2COOH} + H_2O$
Clostridium sporogenes	$\underset{\textit{glycine}}{CH_2NH_2COOH} + \underset{\textit{alanine}}{CH_3CHNH_2COOH} \rightarrow CH_3COOH + \underset{\textit{pyruvic acid}}{CH_3COCOOH} + 2\,NH_3$

2.

$$\underset{\textit{pyruvic acid}}{CH_3{-}\overset{\overset{O}{\|}}{C}{-}COOH} + \underset{\textit{malic acid}}{HOOC{-}\overset{\overset{OH}{|}}{CH}{-}CH_2{-}COOH} \rightleftharpoons \underset{\textit{lactic acid}}{CH_3{-}\overset{\overset{OH}{|}}{CH}{-}COOH} + \underset{\textit{oxalacetic acid}}{HOOC{-}\overset{\overset{O}{\|}}{C}{-}CH_2{-}COOH}$$

terotrophs, but acids such as succinic and lactic acids, the fatty acids, and the monohydroxy alcohols are also oxidized by many different bacteria. Most bacteria can use several different compounds as the main source of energy for metabolism and growth, some as many as a hundred. A few, however, have specific energy requirements. Thus, *Clostridium kluyveri* grows only when it can oxidize ethanol, while *Nitrosomonas europaea* must oxidize ammonia.

Mechanism of Biological Oxidation. Bacteria actually do not obtain energy from the substance oxidized in a single step, as suggested by the equations of table (1). These equations merely represent the over-all process of biological oxidation and are the summation of many separate, consecutive oxidation-reductions, each catalyzed by specific respiratory enzymes. Consider the general equation for aerobic oxidation just written. In most bacteria, such a reaction involves at least the steps shown in 3 below.

Such a stepwise oxidation allows the cell to utilize the energy released in the oxidation in the most efficient manner possible and is characteristic of the reactions of intermediary metabolism in general. The chemical nature and detailed function of the respiratory enzymes involved will be considered later, but it should be noted here that:

(1) Each respiratory enzyme exists in both an oxidized and a reduced state.

(2) The respiratory enzymes enter into reversible oxidation-reduction reactions but are not used up in the over-all process. They function catalytically as *intermediate hydrogen carriers.*

(3) The enzyme that oxidizes the energy source (the *substrate*) cannot directly transfer the hydrogen and electrons of the substrate to oxygen, while the enzyme that finally does react with oxygen cannot oxidize the substrate. Therefore, the functioning of the complete chain of respiratory enzymes is essential for aerobic oxidation.

In most aerobic organisms this system is complete and functional, but in facultative and obligate anaerobes, portions of the hydrogen transport chain may be absent or nonfunctional (see below). In anaerobic respiration, the hydrogen and electrons of substrates are not transferred to molecular oxygen but to other substrates instead. The iron-porphyrin catalysts are not involved in anaerobic respiration, but the flavoproteins may sometimes act as intermediate carriers.

RESPIRATORY ENZYMES IN BACTERIA[17]

Only the respiratory enzymes of higher animals and yeasts have been studied in great detail. However, bacteria definitely contain the same types of enzymes, and there is no reason to doubt that their general properties are also the same.

Dehydrogenases. Dehydrogenases consist of a specific protein, or *apoenzyme,* combined with a reversibly oxidized and reduced *coenzyme.* They oxidize metabolites and are in turn reoxidized by other enzymes, not by molecular oxygen. Dehydrogenases possess a high degree of specificity for the substrate they oxidize and are usually named on the basis of their substrate specificity, *i.e.* lactic dehydrogenase, suc-

---

3.

(1) $A\text{-}H_2$ + pyridinoprotein $\rightleftharpoons$ A + pyridinoprotein-H_2
(2) pyridinoprotein-H_2 + flavoprotein $\rightleftharpoons$ pyridinoprotein + flavoprotein-H_2
(3) flavoprotein-H_2 + 2 cytochrome $Fe^{+++} \rightleftharpoons$ flavoprotein + 2 cytochrome Fe^{++} + 2 H
(4) 2 cytochrome Fe^{++} + 2 cytochrome oxidase $Fe^{+++} \rightleftharpoons$
2 cytochrome Fe^{+++} + 2 cytochrome oxidase Fe^{++}
(5) 2 cytochrome oxidase Fe^{++} + $\frac{1}{2}$ O_2 + 2 $H^+ \rightleftharpoons$ 2 cytochrome oxidase Fe^{+++} + H_2O

The sum of these reactions is $A\text{-}H_2 + \frac{1}{2}\ O_2 \rightarrow A + H_2O$.

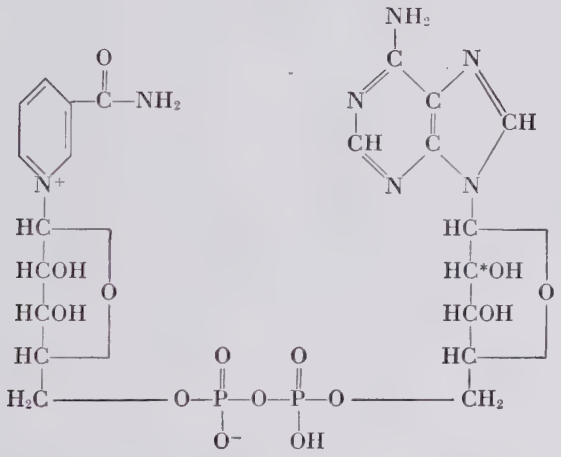
cinic dehydrogenase, etc. This substrate specificity is a function of the protein component, since a single coenzyme may be a part of several different dehydrogenases. The specific protein combines reversibly with both coenzyme and substrate, and the actual oxidation-reduction probably occurs while all three are in physical combination.

Two general types of dehydrogenases are found in bacteria and in other organisms. The *pyridinoproteins* are dehydrogenases containing either diphosphopyridine or triphosphopyridine nucleotide (DPN or TPN) as coenzymes.[29] The provisional structural formula of DPN (coenzyme I, cozymase) is shown in 4 below. The structure of TPN (coenzyme II) is identical with that of DPN except for the presence of an additional molecule of phosphoric acid as shown in 4 below. Only the pyridine ring of both DPN and TPN undergoes reversible oxidation-reduction as indicated in the accompanying equation (5).

The pyridinoproteins are soluble, extracted from cells with relative ease, and their reduced forms are reoxidized by flavoproteins. In bacteria, the malic, lactic, and 3-phosphoglyceraldehyde dehydrogenases are DPN-proteins, while glutamic acid dehydrogenase is a TPN-protein. Many other bacterial dehydrogenases are also pyridinoproteins.

The *cytochrome-linked* dehydrogenases are associated with the insoluble particles of the cell. They were once thought to transfer the hydrogen of their substrates directly to the cyto-

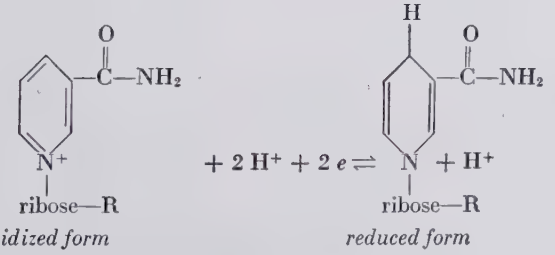
4.



diphosphopyridine nucleotide

*position of third TPN-phosphate

5.



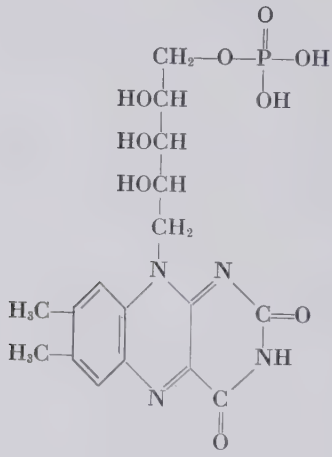
oxidized form *reduced form*

chrome system, hence their name. However, these enzymes are not single enzymes, but a complex of them. For example, succinic dehydrogenase, the most completely characterized complex of this nature, consists of at least the following components: (1) an iron-flavoprotein which oxidizes succinic acid to fumaric acid, (2) Slater's factor, (3) cytochrome *c*–linking factor, and (4) cytochrome *b*. These components react in a sequence, as yet not completely established, to transfer electrons from succinic acid to cytochrome *c* with the net result that succinic acid is oxidized and cytochrome *c* is reduced. Other microbial enzymes of a similar nature are the lactic dehydrogenase of yeast and the formic dehydrogenase of *Escherichia coli*.

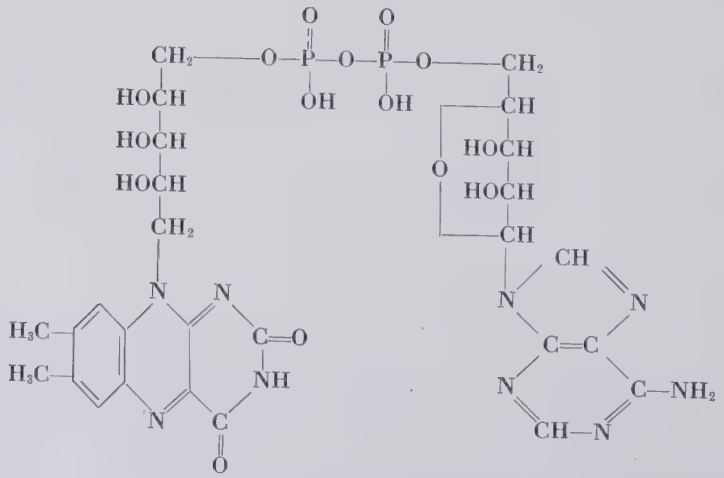
Flavoproteins.[6, 18] The flavoproteins are respiratory enzymes containing either riboflavin phosphate or flavin adenine dinucleotide as coenzymes. (See **6** below.)

In both riboflavin coenzymes, oxida-

6.



riboflavin phosphate



flavin adenine dinucleotide

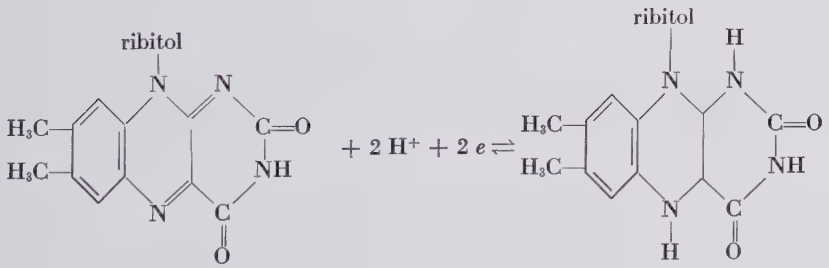
tion-reduction is confined to the flavin portion of the molecule. (See **7** below.) Many flavoproteins contain one or more atoms of iron, molybdenum, or copper. The metals apparently facilitate the transfer of electrons from the reduced flavin coenzyme to the cytochrome system.

Derivatives of riboflavin are present in all bacteria and are found in especially large amounts in some anaerobes. Some flavoproteins oxidize substrates with the formation of hydrogen peroxide (**8** below). It is generally believed that such flavoprotein *oxidases* are usually responsible for the formation of hydrogen peroxide in bacteria. Other flavoproteins also oxidize substrates but transfer electrons to oxygen by way of the cytochrome system. Still others function in the chain of electron transport by oxidizing reduced pyridine nucleotides and then reducing cytochrome. These enzymes are called *cytochrome reductases* or *diaphorases*. Flavoproteins of all three types are of apparently general occurrence in bacteria.

Iron-porphyrin Protein Enzymes.[55] Several important respiratory enzymes have iron-porphyrin compounds as coenzymes. These iron-porphyrins are closely related to, but not identical with, the heme of hemoglobin. Only the iron atom of the iron-porphyrin undergoes reversible oxidation-reduction. Cells of higher organisms contain at least three iron-porphyrin protein pigments, the *cytochromes a, b,* and *c,* and other similar proteins. The cytochromes are usually, but not always, present in aerobic bacteria and absent in obligate anaerobes. Bacterial cytochromes differ from those of plant and animal tissues in absorption spectra, specificity, and other physical and chemical characteristics. Bacterial cytochromes are remarkable for their variety, both within a single species and among different species. The cytochromes take part in electron transport by accepting electrons from cytochrome reductase and passing them on to cytochrome oxidase.

Cytochrome oxidase, Warburg's respiratory enzyme, is a portion of the submicroscopic particulate structure of the cell, but it may be brought into solution or stable suspension by treatment with ultrasonic vibrations. It is also an iron-porphyrin protein. Cytochrome oxidase is the enzyme which reduces molecular oxygen to H_2O to complete the last link in the chain of aerobic respiration. The identity and number of cytochromes functioning between flavoprotein and

7.



oxidized form *reduced form*

8.

$$\text{substrate-}H_2 + \text{flavoprotein} \rightarrow \text{substrate} + \text{flavoprotein-}H_2$$
$$\text{flavoprotein-}H_2 + O_2 \rightarrow \text{flavoprotein} + H_2O_2$$

cytochrome oxidase is not known for most bacteria. More than one oxidase may be present in a single cell.

A few bacteria, such as *Streptococcus fecalis* and *Lactobacillus delbruekii,* carry out aerobic oxidations in the complete absence of cytochromes or cytochrome oxidase. In these organisms, the respiratory enzyme that transfers electrons to molecular oxygen is flavoprotein.[18]

Most organisms contain an enzyme called *catalase* which decomposes hydrogen peroxide.

$$2\,H_2O_2 \rightarrow 2\,H_2O + O_2$$

The catalase crystallized from beef liver is an iron-porphyrin protein, and it has been generally accepted that catalase from other sources has a similar chemical nature, but recent evidence suggests that catalases other than iron-porphyrins may be present in some bacteria. Catalase is present in almost all aerobic bacteria and some facultative anaerobes, but under usual conditions of culture it cannot be detected in clostridia and some of the streptococci, lactic acid bacteria, and dysentery bacteria.

The *peroxidases* are enzymes, usually iron-porphyrin proteins but sometimes flavoproteins, which catalyze the oxidation of substrates by hydrogen peroxide. Their distribution in bacteria parallels that of the cytochromes and catalase, and their function in bacterial metabolism is uncertain.

THE RELATION OF BACTERIA TO MOLECULAR OXYGEN

The bacteria differ from one another in their relationship to molecular oxygen. Certain bacteria, the *obligate aerobes,* require ready access to air, growing feebly or not at all in its absence. Such organisms may lack certain respiratory enzymes necessary for anaerobic respiration, or the end products of their anaerobic metabolism may be toxic to them. The exact cause of their obligate oxygen requirement remains unknown. This group includes such well-known bacteria as the nitrifying bacteria, some of the sulfur bacteria, *Bacillus subtilis* and related forms, Azotobacter, the diphtheria bacillus, the cholera vibrio, and others.

However, most bacteria can grow in the complete or virtual absence of molecular oxygen. Two types of anaerobic bacteria may be distinguished. The most numerous are the *facultative anaerobes,* which respire with equal facility in the presence or absence of molecular oxygen. In broth cultures the dissolved oxygen is soon exhausted, and, after a preliminary period of aerobic growth, the culture continues to develop under essentially anaerobic conditions. The *obligate anaerobes* are unable to grow in the presence of molecular oxygen, and oxygen is actively toxic to them; vegetative cells die very quickly upon exposure to air, although spores are highly resistant. Members of the genus Clostridium, together with certain micro-aerophilic forms which tolerate very low concentrations of oxygen, are usually included in this group.

Bacteria are unexcelled in the possession of powerful and varied means for obtaining energy from nutrients in the absence of oxygen. The energy obtained from a given quantity of nutrients is usually much less under anaerobic conditions than in the presence of oxygen, because oxidation of substrates is less complete, and anaerobic metabolism is characterized by a rapid utilization of oxidizable substrates and a great accumulation of partially oxidized end products.

Bacteria perform energy-yielding oxidations anaerobically by using both inorganic and organic compounds as hydrogen acceptors instead of molecular oxygen. Thus, *E. coli* contains respiratory enzymes which couple the oxidation of lactic acid with the reduction of nitrate. (See 9 on opposite page.)

Bacteria also frequently couple oxidation of one organic compound with the reduction of a second. In this manner,

glyceraldehyde may be oxidized by the enzymatic transfer of hydrogen to pyruvic acid. (See **10** below.)

This type of reaction is the chief mode of oxidation-reduction in the fermentation of carbohydrates, the chief anaerobic energy source for the great majority of bacteria. However, certain of the obligate anaerobes possess only a very limited ability to metabolize carbohydrate, and an important source of energy for these bacteria appears to be the coupled oxidation-reduction of pairs of amino acids.

As already mentioned, the obligate anaerobes contain none or very little of the iron-porphyrin respiratory enzymes, a definite indication of enzymatic deficiency. However, the toxicity of molecular oxygen to these bacteria requires more explanation than their lack of oxygen-transporting enzymes. The catalase theory is based on the discovery that certain bacteria produce hydrogen peroxide in amounts sufficient to be toxic to themselves. In cultures of those species which do not produce catalase and are peroxide-sensitive, peroxide accumulates and the culture becomes self-sterilizing; the pneumococcus is an example of this type of microorganism. Those bacteria which produce catalase decompose the peroxide as it forms, and it does not accumulate to toxic concentrations. The obligate anaerobes are peroxide-sensitive and do not form catalase. Hence it has been suggested that these organisms form peroxide rapidly in the presence of air and therefore oxygen is toxic to them. Although this theory is accepted by some, it entirely lacks supporting evidence, for all attempts to demonstrate peroxide in cultures exposed to air have failed. It is not clear why oxygen is toxic for the obligate anaerobes. Possibly some of the respiratory enzymes of the obligate anaerobes are oxygen-labile, *i.e.*, active in the reduced state and inactive in the oxidized state, as are some of the bacterial hemolysins or hydrogenase, or perhaps the substrates of some of the enzymes of these organisms are unstable under aerobic conditions.

CONSERVATION AND TRANSFER OF THE ENERGY RELEASED IN BIOLOGICAL OXIDATIONS[22, 50]

So far nothing has been said of how energy released in respiration is conserved and made available for synthetic purposes. Yet these processes are as important as the energy-yielding reactions themselves, since energy simply liberated as heat (as in oxidation carried out in the test tube by the organic chemist) is not available for synthesis of new protoplasm.

The general principles involved are well illustrated by a reaction already discussed, the coupled oxidation-reduction of glyceraldehyde and pyruvic acid. Our present concepts of energy transfer in biological systems arose from detailed studies of this key reaction in carbohydrate fermentation (see below). Actually several different enzymes and coenzymes participate in this complicated reaction,

9.

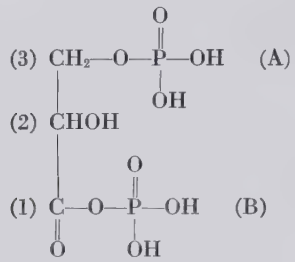
$$\underset{\textit{lactic acid}}{CH_3\text{-}CHOH\text{-}COOH} + HNO_3 \rightleftharpoons \underset{\textit{pyruvic acid}}{CH_3\text{-}CO\text{-}COOH} + HNO_2 + H_2O$$

10.

$$\underset{\textit{glyceraldehyde}}{CH_2OH\text{-}CHOH\text{-}CHO} + \underset{\textit{pyruvic acid}}{CH_3\text{-}CO\text{-}COOH} + H_2O \rightleftharpoons \underset{\textit{glyceric acid}}{CH_2OH\text{-}CHOH\text{-}COOH} + \underset{\textit{lactic acid}}{CH_3\text{-}CHOH\text{-}COOH}$$

but for the purposes at hand, it may be represented as shown in **11**. Note that both inorganic and organic forms of orthophosphoric acid (H_3PO_4) participate in the reaction. This is because the inorganic and organic phosphate compounds form an integral part of the energy-conserving and transferring mechanism.

The key to the role of phosphate in energy metabolism lies in the difference between the two kinds of phosphate groups present in 1, 3-diphosphoglyceric acid. Let us see what this difference is and wherein lies its significance for biological oxidation.

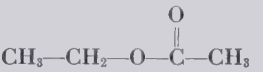


1,3-diphosphoglyceric acid

Phosphate Group A

(1) The phosphate group is united with the rest of the molecule through an ester linkage with a primary alcohol group.

(2) By analogy with simple and familiar organic compounds, this linkage may be compared with the ester bond joining ethanol and acetic acid in ethyl acetate.



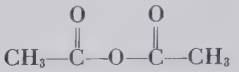
ethyl acetate

(3) Like ethyl acetate, this *phosphate ester* is not easily hydrolyzed and liberates but little heat (energy) on hydrolysis.

(4) For these reasons, organic phosphate esters, as well as inorganic phosphate salts, are said to contain *low-energy phosphate bonds,* which are often designated by the shorthand notation, R–P.

Phosphate Group B

(1) The phosphate group is united with the rest of the molecule through an anhydride linkage to a carboxyl group.



acetic anhydride

(2) By analogy, this bond may be compared to the anhydride bond between two molecules of acetic acid in acetic anhydride.

(3) Like acetic anhydride, this *phosphate anhydride* is readily hydrolyzed and liberates large amounts of heat (energy) on hydrolysis.

(4) Organic phosphate anhydrides are said to contain *high-energy phosphate bonds,* which are represented as R~P.

When the oxidation of 3-phosphoglyceraldehyde is coupled with the uptake of inorganic phosphate to form 1, 3-diphosphoglyceric acid, the energy liberated as heat is very much less than the heat released in the simple oxidation of glyceraldehyde to glyceric acid. The reason for this difference should now be apparent: the missing energy has been trapped and conserved in the form of the high-energy phosphate bond of 1, 3-diphosphoglyceric acid. The detailed reaction mechanism is shown in **12** on opposite page. All three steps are catalyzed by 3-phosphoglyceraldehyde dehydrogenase. 3-phosphoglyceraldehyde first combines with the thiol (SH) group of the enzyme to form a thiohemiacetal which is oxidized to a thioacyl group with DPN as the hydrogen acceptor. Finally in step (*c*), the thioacyl group is cleaved by the addition of inorganic phosphate to yield 1, 3-diphosphoglyceric acid and to regenerate the dehydrogenase in its SH form. Such a coupling of oxidation with the uptake of low-energy inorganic phosphate into high-energy organic phos-

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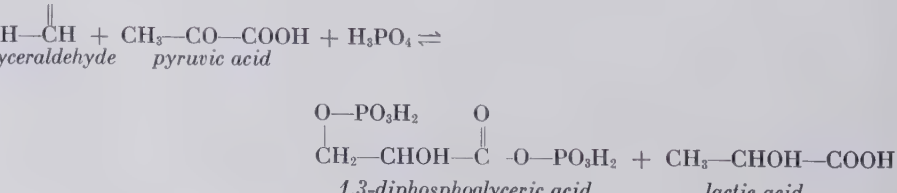
11.



phate compounds appears to be the chief method of energy conservation in all living cells. Therefore, representation of the general reaction of biological oxidation may be broadened to include the energy-conserving mechanism:

$$A{-}H_2 + B + P \rightleftharpoons \underset{\text{(inorganic)}}{A{\sim}P} + \underset{\text{(high-energy)}}{B{-}H_2}$$

There remains another problem—how the energy conserved in high-energy phosphate bonds is transferred to the appropriate place in the metabolic machinery where it may be used up in energy-requiring syntheses. Using the same example as before, this is what occurs:

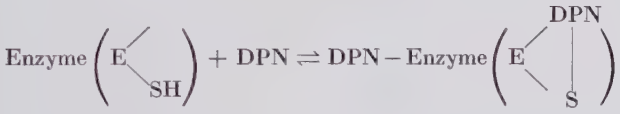
1,3-diphosphoglyceric acid + adenosine diphosphate ⇌
3-phosphoglyceric acid + adenosine triphosphate

Adenosine diphosphate (ADP) and adenosine triphosphate (ATP) have the structure shown in **13** below.

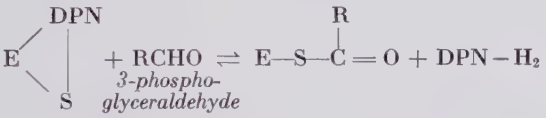
Note that ATP has two energy-rich bonds while ADP has only one, which is not usually active in energy transfer. This is the only difference between the

12.

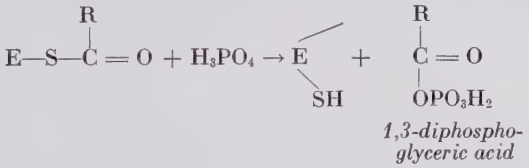
(*a*)



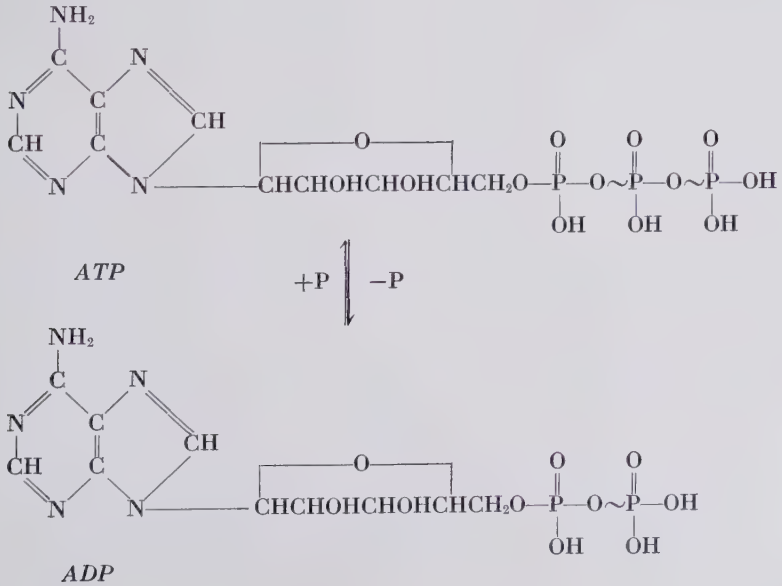
(*b*)



(*c*)



13.



two substances. All cells appear to have enzymes which reversibly convert one to the other by the addition or removal of an energy-rich bond. The resemblance of this reversible system to the reversibly oxidized and reduced hydrogen carriers is very striking. By analogy they may be called intermediate *energy carriers* or *energy donors* and *energy acceptors,* and this is exactly how they function in metabolism. ATP and ADP form the connecting link between the energy-yielding reactions of respiration and the energy-demanding reactions of synthesis. (See **14** below.)

In subsequent sections, it will be seen that many types of oxidations, aerobic and anaerobic, inorganic and organic, generate ATP and that the energy stored in ATP is used in almost every type of biological synthesis. High-energy phosphate bonds are generated, not only during oxidations at the substrate level, but also in the oxidations accompanying the further steps in aerobic respiration. The steps in electron transfer implicated in oxidative phosphorylation are the reoxidation of DPN-H_2 and reduced cytochrome.

Racker[50] has classified metabolic reactions that lead to the formation of ATP into five types:

(1) Substrate level oxidative phosphorylation
Example: Oxidation of 3-phosphoglyceraldehyde
(2) Phosphorolysis of C–C, C–N, or C–S bonds
Examples: C–C: phosphoroclastic breakdown of pyruvic acid
C–N: phosphorolysis of citrulline
C–S: phosphorolysis of acetyl-coenzyme A
(3) Dehydration of 2-phosphoglyceric acid to phosphoenolpyruvic acid
(4) Oxidative phosphorylation
(5) Photosynthetic phosphorylation

All these reactions either have been or will be discussed in appropriate sections of this chapter.

CARBOHYDRATE METABOLISM

Carbohydrates serve a dual function in the metabolism of heterotrophic bacteria: (1) they are oxidized as the chief source of energy in most species and (2) they are the principal, and often the only, source of carbon for the synthesis of bacterial carbohydrates, lipids, amino acids, purine and pyrimidine bases, and all other organic cell constituents. In practice, these functions are inseparable. The metabolic transformations necessary for the assimilation of carbohydrates into cell material are often also energy-yielding breakdowns. In addition, almost all the reactions involved in the degradation of carbohydrates are metabolically reversible and thus are also used for synthesis of new carbohydrates from small molecules such as CO_2, acetic acid, and ethanol.

Bacteria form a bewildering variety of products from carbohydrates, but their metabolism proceeds along a relatively few well-defined pathways of wide distribution in bacteria and in all other living things as well. Therefore variation in

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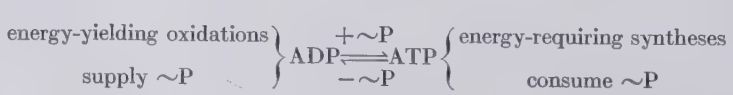
14.



metabolism among different species is not so much the result of fundamental differences in mode of metabolism as of differences in the relative importance of the several possible common pathways.

METABOLISM OF COMPLEX CARBOHYDRATES[53]

Carbohydrates composed of two or more monosaccharide units linked together by *glycosidic bonds* are called complex sugars. Those with a definite small number of monosaccharide units are called *oligosaccharides*; most of them are *disaccharides*, such as maltose which is composed of two molecules of glucose. *Polysaccharides* contain a large and indefinite number of monosaccharide units. For example, the amylose fraction of starch contains molecules made up of straight chains of 300 to 1000 glucose units.

Hydrolysis of Complex Sugars. Although free monosaccharides are widely distributed in nature, by far the greater bulk of naturally occurring carbohydrates are oligosaccharides and polysaccharides. With few exceptions bacteria must first break down the complex sugars into their constituent monosaccharides before they can be used as energy sources or as raw material for the synthesis of new protoplasm. This is done by cleaving the glycosidic bond uniting two monosaccharide units.

Bacteria break down complex sugars by three principal mechanisms, all of which may be illustrated with one sugar, sucrose. The examples in **15** give both the reaction and a typical enzyme which catalyzes it.

As will be seen in the section immediately following, the last two kinds of enzymes may also synthesize oligosaccharides and polysaccharides. However, hydrolysis of compound sugars is a virtually irreversible process and plays no important part in their synthesis. Nevertheless, bacteria possess a formidable battery of hydrolytic enzymes which are of great importance to the bacterium in utilizing the carbohydrate foodstuffs of its environment. They are of equal importance to the bacteriologist in classifying, identifying, and differentiating bacteria, because the presence or absence of enzymes hydrolyzing complex sugars largely determines the fermentation reactions of different species. Some of these enzymes are synthesized by the bacterial cell whether their substrate is present or not (*constitutive enzymes*), while others are produced only in the presence of the appropriate substrate (*induced enzymes*).

Although *cellulose* is the most widely distributed polysaccharide in nature, the ability to bring about its decomposition is possessed by only a few kinds of organisms. The enzyme cellulase is found in a few higher organisms, some snails, and a few marine forms, but even the herbivorous animals are dependent upon the

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Breakdown of Complex Sugars

15.

(1) *Hydrolysis*

$$\text{sucrose} + H_2O \xrightarrow{\text{invertase}} \text{glucose} + \text{fructose}$$

(2) *Phosphorolysis*

$$\text{sucrose} + H_3PO_4 \xrightarrow[\text{phosphorylase}]{\text{sucrose}} \text{glucose-1-phosphate} + \text{fructose}$$

(3) *Transglycosidation*

$$n\ \text{sucrose} \xrightarrow[\text{sucrase}]{\text{dextran}} (\text{anhydroglucose})_n + n\ \text{fructose}$$

dextran

presence of cellulose-decomposing microorganisms in the intestine for the preliminary decomposition of this polysaccharide. The breakdown of cellulose and other polysaccharides by bacteria is an essential part of the decomposition of plant residues and the consequent returning to the atmosphere of carbon dioxide removed through the photosynthetic activities of chlorophyll-containing plants. Relatively few species of bacteria are able to decompose cellulose, but those that do are widely distributed in the soil and the mud of sea bottoms as well as in the intestines of herbivorous animals.

Cellulose is hydrolyzed to glucose, sometimes directly, and sometimes with the intermediate formation of the disaccharide cellobiose. The enzymes attacking cellulose may be secreted extracellularly or may be attached to the surface of the cellulose-decomposing bacterial cell.

Unlike cellulose, *starch* may be hydrolyzed by a wide variety of living organisms, and the extracellular enzyme diastase, or amylase, is possessed by many bacteria. Like cellulose, however, starch is hydrolyzed in at least two stages, first to the disaccharide maltose, and second by a further hydrolysis to glucose.

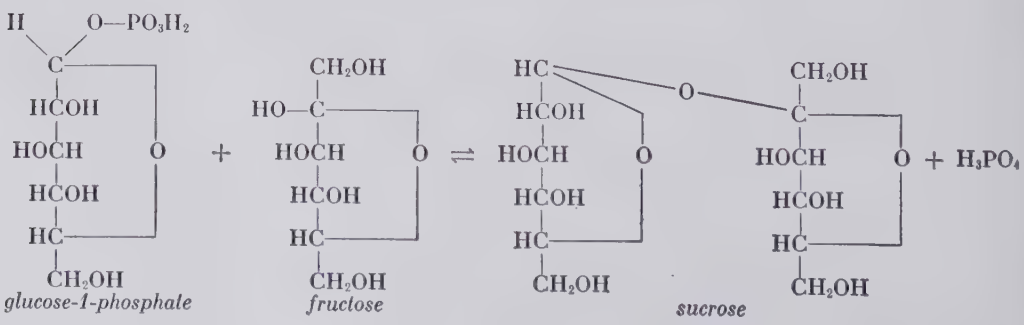
Many *disaccharides* are also hydrolyzed by bacterial enzymes; sucrose by invertase, maltose by maltase, cellobiose by cellobiase, lactose by β-galactosidase, etc.

Synthesis of Complex Sugars.[23] The essential reaction in the synthesis of oligosaccharides and polysaccharides is the formation of the glycosidic bond. This reaction may be studied in its simplest form in the synthesis of disaccharides. Sucrose is formed from glucose-1-phosphate and fructose by the action of the enzyme sucrose phosphorylase found in *Pseudomonas saccharophila, Leuconostoc mesenteroides* and other bacteria (see **16**).

In contrast to the *hydrolysis* of sucrose by invertase, the *phosphorolysis* of sucrose by sucrose phosphorylase is reversible, and this enzyme readily synthesizes the disaccharide. This is because it is very hard to eliminate a molecule of water from the two monosaccharides in aqueous solution, while it is relatively easy to split out a molecule of phosphoric acid. Although sucrose phosphorylase is specific in its requirement for glucose-1-phosphate, other sugars such as sorbose, arabinose, and others, may be substituted for fructose. Thus, the one enzyme can synthesize a number of different disaccharides.

In syntheses catalyzed by enzymes of the sucrose phosphorylase type, the problem of glycosidic bond synthesis becomes one of glucose-1-phosphate production. The formation of glucose-1-phosphate and its utilization for disac-

16.



charide synthesis is a good example of how the energy stored in ATP by oxidative reactions is used for synthetic purposes (**17** below). Once glycosidic linkages are formed, other complex sugars may be formed by the transfer of monosaccharide units, or *transglycosidation.*[15] Again, this mechanism may be illustrated with sucrose:

$$\underset{(\textit{sucrose})}{\text{glucosido-fructoside}} + \text{sorbose} \rightleftharpoons \text{glucosido-sorboside} + \text{fructose}$$

Note that the *number* of glycoside linkages remains the same. The glucose unit has merely been transferred from glycosidic linkage with fructose to glycosidic linkage with sorbose—hence, transglycosidation. It is interesting that this reaction is catalyzed by sucrose phosphorylase, which is therefore both a phosphorylase and a transglycosidase.

Other transglycosidases catalyze the formation of polysaccharides. Thus, the dextran sucrase of *Leuc. mesenteroides* forms from sucrose the polysaccharide dextran, which is composed of many glucose units.

$$n \text{ sucrose} \rightarrow \underset{\textit{dextran}}{\text{anhydroglucose}_n} + \text{fructose}$$

Note that here again the number of glycosidic bonds in the newly formed dextran is equal to the number of glycosidic linkages in the sucrose which was broken down. Similar transglycosidases are the levan sucrase of *B. megaterium,* which forms the fructose polysaccharide levan from sucrose, and the amylomaltase of *E. coli* which forms a starchlike polysaccharide from maltose. The ability to catalyze reactions of transglycosidation is a widely distributed enzymic property. Even enzymes such as invertase and β-galactosidase, long thought to have only a hydrolytic action, have now been shown to possess transglycosidase activity as well. The reactions of transglycosidation apparently proceed without the intermediate formation of glucose-1-phosphate, but since the only known mechanism whereby sucrose or other disaccharides are synthesized by bacteria involves glucose-1-phosphate and phosphorylases, it is likely that the ultimate energy source for transglycosidation is also the high-energy phosphate bonds of ATP.

Another type of transglycosidation involves the participation of a derivative of the pyrimidine uracil, uridine diphosphate glucose (UDPG) (**18**), as the monosaccharide unit donor. Yeast contains an enxyme which synthesizes sucrose according to the equation:

$$\text{UDPG} + \text{fructose} \rightleftharpoons \text{sucrose} + \text{UDP}$$

UDPG is regenerated as follows:

$$\text{UDP} + \text{glucose-1-P} \rightleftharpoons \text{UDPG} + \text{P}$$

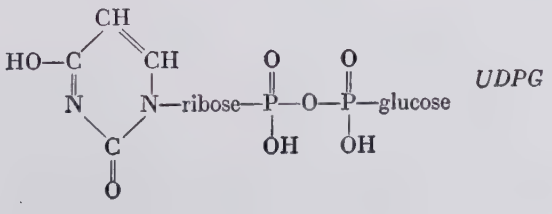
17.

$$\text{glucose} + \text{ATP} \rightarrow \text{glucose-6-phosphate} + \text{ADP}$$
$$\text{glucose-6-phosphate} \rightleftharpoons \text{glucose-1-phosphate}$$
$$\text{glucose-1-phosphate} + \text{fructose} \rightleftharpoons \text{sucrose} + H_3PO_4$$

Net reaction:

$$\text{glucose} + \text{fructose} + \text{ATP} \rightarrow \text{sucrose} + \text{ADP} + H_3PO_4$$

18.



UDPG

If the examples discussed here are representative of the mechanisms responsible for complex sugar synthesis in all bacteria, it may be concluded that bacteria do not form oligosaccharides and polysaccharides by directly combining simple monosaccharides. Instead, they (1) form glucose-1-phosphate or analogous compounds with the energy of ATP, (2) form initial glucosidic bonds by splitting out H_3PO_4 between glucose-1-phosphate and another monosaccharide or UDP, and (3) form other disaccharides and polysaccharides by the transglycosidation reaction.

Great progress has been made in recent years in understanding how polysaccharides with more than one kind of monosaccharide unit are synthesized. For example let us consider the biosynthesis of hyaluronic acid, a polysaccharide occurring both in bacteria and in higher animals and of probable importance in certain bacterial infections. Hyaluronic acid is composed of a large number of basic units consisting of a molecule of glucuronic acid and one of N-acetylglucosamine (**19**). It is synthesized as shown in **20** below:

The specific capsular polysaccharides of pneumococcus types I and III are synthesized by analogous mechanisms.

BREAKDOWN OF HEXOSES AND PENTOSES

This topic is most simply presented by considering first the degradation of glucose. Since pentoses are frequent early products of glucose catabolism, the breakdown of pentoses is also discussed here. Finally, the way in which other common hexoses and hexose derivatives are channeled into the pathways of glucose metabolism will be shown.

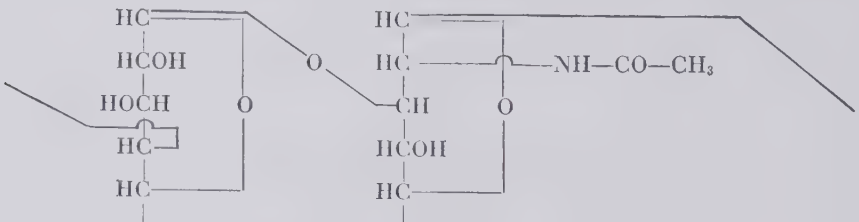
Diagram **21** (on opposite page) outlines the four principal known pathways of glucose breakdown in bacteria. It should be used only as a guide to the detailed discussion of these pathways, for in order to show all possible modes of glucose degradation in a single diagram, certain simplifications have been made and alternate variations within a single pathway have been omitted.

All the mechanisms for breakdown of glucose may be roughly divided into three stages:

(1) Conversion of glucose, by phosphorylation, oxidation, or by both processes, to a 6-carbon sugar which is the substrate for stage 2.

(2) Cleavage of the carbon skeleton of the 6-carbon intermediate formed in stage 1 to two 3-carbon fragments or to

19.



glucuronic acid *N-acetyl-glucosamine*

Basic unit of hyaluronic acid

20.

(a) UDP-glucose + 2 DPN → UDP-glucuronic acid + 2 DPN-H_2

(b) glucose-6-phosphate → fructose-6-phosphate
fructose-6-phosphate + glutamine → glucosamine-6-phosphate + glutamic acid
glucosamine-6-phosphate + acetyl-coenzyme A → N-acetyl-glucosamine-6-phosphate + coenzyme A
N-acetyl-glucosamine-6-phosphate → N-acetyl-glucosamine-1-phosphate
N-acetyl-glucosamine-1-phosphate + UTP → UDP-N-acetyl-glucosamine-1-phosphate + P-P

(c) UDP-N-acetyl-glucosamine-1-phosphate + UDP-glucuronic acid → hyaluronic acid + P + 2 UDP

a 5-carbon sugar (pentose) and CO_2.

(3) Further metabolism of the reaction products of stage 2 to pyruvic acid (note exception in pathway 4).

The fate of pyruvic acid, the common product of all 4 pathways, will be the subject of the section immediately following.

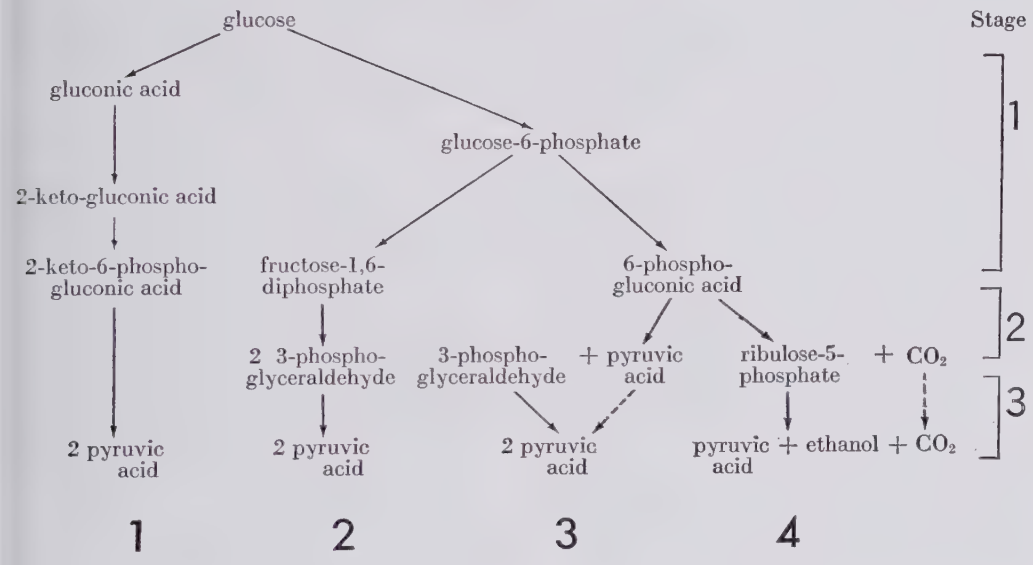
Pathway 1: Nonphosphorylating oxidation of glucose. Originally described in pseudomonads and acetic acid bacteria, these reactions have now been found in many other gram-negative and gram-positive bacteria. The unique step in this mechanism is the direct oxidation, without prior phosphorylation, of glucose to gluconic acid. Further steps vary in different bacteria. An example of the pathway from glucose to pyruvic acid in *Ps. fluorescens* is shown in equation **22**.

It is believed that considerable useful energy may be generated by means of phosphorylations coupled with the oxidation of glucose to 2-ketogluconate.

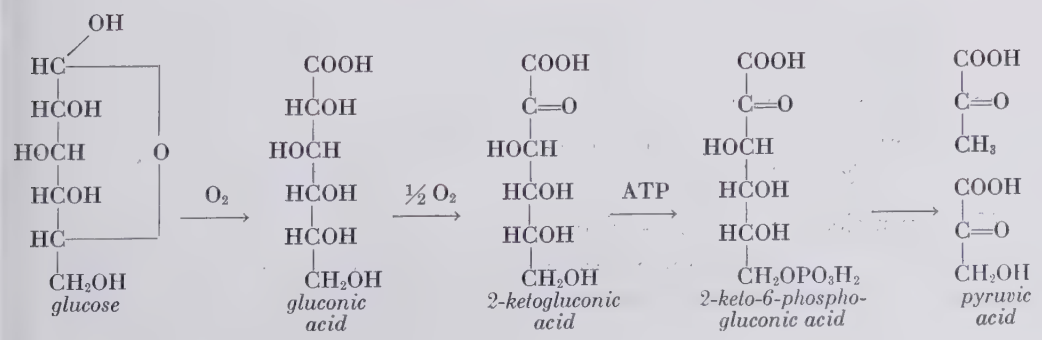
Pathways 2, 3, and 4. Formation of glucose-6-phosphate as a common initial step. Glucose is metabolized by way of the three remaining pathways

21.

Breakdown of Glucose



22.



by being first phosphorylated to glucose-6-phosphate by ATP and the enzyme *hexokinase.*

$$\text{glucose} + \text{ATP} \rightarrow \text{glucose-6-phosphate} + \text{ADP}$$

Pathway 2: Phosphorylating glycolysis.[3, 62] Breakdown of glucose by phosphorylating glycolysis, the Embden-Meyerhof scheme, occurs in many bacteria, sometimes as the sole means of glucose breakdown, as in the homofermentative lactic acid bacteria, but more frequently in combination with other pathways. Glycolysis does not involve the participation of molecular oxygen, although it may occur under both aerobic and anaerobic conditions.

Three basic chemical reactions occur in typical phosphorylating glycolysis as shown in **23.** The chemical nature of the hydrogen acceptor *B* in reaction 2 determines the type of the fermentation. If *B* is pyruvic acid, it is a lactic fermentation; if it is acetaldehyde, the fermentation is alcoholic, etc.

The actual mechanism of phosphorylating glycolysis is complicated by many intermediate steps in which phosphorylated carbohydrates, coenzymes, and enzymes participate. These complications serve to make the pathway from glucose to pyruvic acid almost completely reversible and to conserve efficiently the oxidatively liberated energy in the form of the high-energy phosphate bonds of ATP. The transformation of glucose to pyruvic acid by way of the Embden-Meyerhof scheme may be considered to proceed in four stages. (All the following reactions are given in sequence in diagram **24** on opposite page.)

(1) ***Formation of fructose-1,6-diphosphate.*** The hexose actually split to triose is fructose-1,6-diphosphate, formed as follows:

$$\text{glucose-6-phosphate} \overset{\text{isomerase A}}{\rightleftharpoons} \text{fructose-6-phosphate}$$

$$\text{fructose-6-phosphate} + \text{ATP} \xrightarrow{\text{phosphohexokinase}} \text{fructose-1,6-diphosphate} + \text{ADP}$$

(2) ***Cleavage of fructose-1,6-diphosphate to triose.*** Fructose-1,6-diphosphate is split into two trioses, which are enzymatically interconvertible.

$$\text{fructose-1,6-diphosphate} \overset{\text{aldolase}}{\rightleftharpoons} \text{3-phosphoglyceraldehyde} + \text{dihydroxyacetone phosphate}$$

$$\text{3-phosphoglyceraldehyde} \overset{\text{isomerase B}}{\rightleftharpoons} \text{dihydroxyacetone phosphate}$$

The occurrence of this aldolase reaction may be considered the unique reaction of the Embden-Meyerhof pathway.

(3) ***Oxidation of 3-phosphoglyceraldehyde.*** Of the two trioses formed in stage 2, only 3-phosphoglyceraldehyde is oxidized under normal conditions. The reaction is catalyzed by 3-phosphoglyceraldehyde dehydrogenase which contains DPN as a coenzyme. The detailed mechanism of this reaction has already been described.

$$\text{3-phosphoglyceraldehyde} + \text{DPN} + \text{H}_3\text{PO}_4 \rightleftharpoons \text{1,3-diphosphoglyceric acid} + \text{DPN—H}_2$$

23.

(1) Cleavage of hexose to two trioses

$$\underset{\textit{glucose}}{\text{CHO—(CHOH)}_4\text{—CH}_2\text{OH}} \rightarrow \underset{\textit{glyceraldehyde}}{\text{CH}_2\text{OH—CHOH—CHO}} + \underset{\textit{dihydroxyacetone}}{\text{CH}_2\text{OH—CO—CH}_2\text{OH}}$$

(2) Oxidation of aldehyde to carboxylic acid

$$\underset{\textit{glyceraldehyde}}{\text{CH}_2\text{OH—CHOH—CHO}} + \text{B} + \text{H}_2\text{O} \rightarrow \underset{\textit{glyceric acid}}{\text{CH}_2\text{OH—CHOH—COOH}} + \text{B—H}_2$$

(3) Intramolecular oxidation-reduction of hydroxyl groups

$$\underset{\textit{glyceric acid}}{\text{CH}_2\text{OH—CHOH—COOH}} \rightarrow \underset{\textit{pyruvic acid}}{\text{CH}_3\text{—CO—COOH}} + \text{H}_2\text{O}$$

The high-energy phosphate group of 1,3-diphosphoglyceric acid is transferred to ADP under the influence of another enzyme:

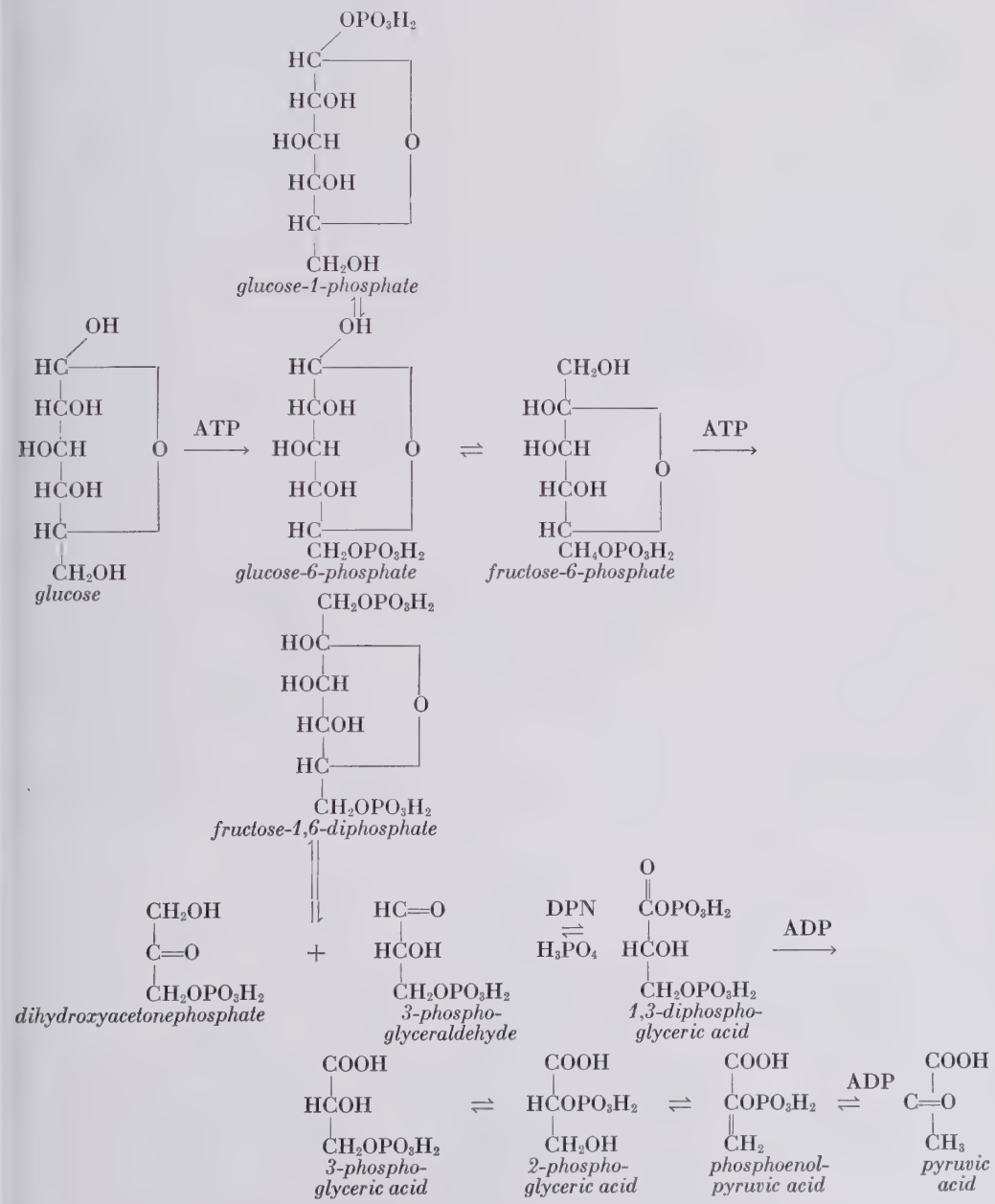
1,3-diphosphoglyceric acid + ADP $\rightleftharpoons$ 3-phosphoglyceric acid + ATP

Finally, the reduced DPN is reoxidized by an appropriate hydrogen acceptor and its dehydrogenase. In the lactic fermentation, the reaction is:

pyruvic acid + DPN—H_2 $\overset{\text{lactic dehydrogenase}}{\rightleftharpoons}$ lactic acid + DPN

24.

Phosphorylating Glycolysis of Glucose to Pyruvic Acid



(4) ***Formation of pyruvic acid.*** 3-Phosphoglyceric acid is rearranged to 2-phosphoglyceric acid:

$$\text{3-phosphoglyceric acid} \xrightleftharpoons[\text{mutase}]{\text{triose}} \text{2-phosphoglyceric acid}$$

Then 2-phosphoglyceric acid undergoes an intermolecular oxidation-reduction to phosphopyruvic acid:

$$CH_2OH{-}\underset{\displaystyle OPO_3H_2}{\underset{|}{C}}{-}COOH \xrightleftharpoons{\text{enolase}} CH_2{=}\underset{\displaystyle O\sim PO_3H_2}{\underset{|}{C}}{-}COOH + H_2O$$

The formation of phosphopyruvic acid is another example of how energy released in oxidations is conserved in high-energy phosphate bonds. Here the low-energy phosphate group of 2-phosphoglyceric acid is converted into the high-energy group of phosphopyruvic acid. This group may be considered to be formed by the elimination of a molecule of water between phosphoric acid and the highly acidic enolic hydroxyl group of enol-pyruvic acid; *i.e.,* it is a phosphate anhydride.

In the final pyruvate-forming reaction, the high-energy bond of phosphopyruvate is enzymatically transferred to ADP.

$$\text{phosphopyruvic acid} + \text{ADP} \rightleftharpoons \text{pyruvic acid} + \text{ATP}$$

Since many bacteria such as the homofermentative lactobacilli gain their energy from anaerobic oxidation of glucose to lactate, there must be a net production of energy in the process. A balance sheet in loss and gain of ATP makes this clear.

ATP used up per mole of glucose
- 1 ATP used for formation of glucose-6-phosphate from glucose
- 1 ATP used for formation of fructose-1,6-diphosphate from fructose-6-phosphate

Total 2 ATP used up

ATP formed per mole of glucose
- 2 ATP formed from oxidation of 3-phosphoglyceraldehyde to 3-phosphoglyceric acid (1 glucose → 2 glyceraldehyde)
- 2 ATP formed from transformation of 2-phosphoglyceric acid to phosphopyruvic acid.

Total 4 ATP formed

Therefore, oxidation of one molecule of glucose to lactic acid results in the net formation of two moles of ATP.

Pathways 3 and 4: Formation of 6-Phosphogluconic Acid as a Common Step. Glucose-6-phosphate is oxidized to 6-phosphogluconic acid by dehydrogenases which, in different organisms, may require either TPN or DPN as coenzyme. (See **25.**)

Pathway 3:[62] **The Entner-Doudoroff Pathway.** In many gram-negative bacteria, 6-phosphogluconic acid is cleaved to two 3-carbon compounds through the intermediate formation of 2-keto-3-deoxy-6-phosphogluconic acid (KDPG). Thus, splitting of this compound identifies the Entner-Doudoroff

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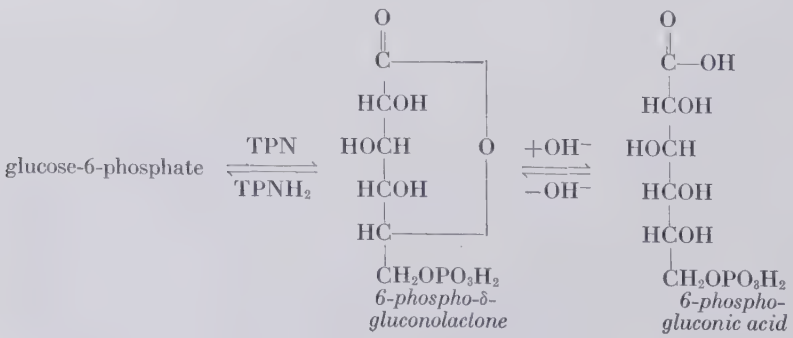
25.

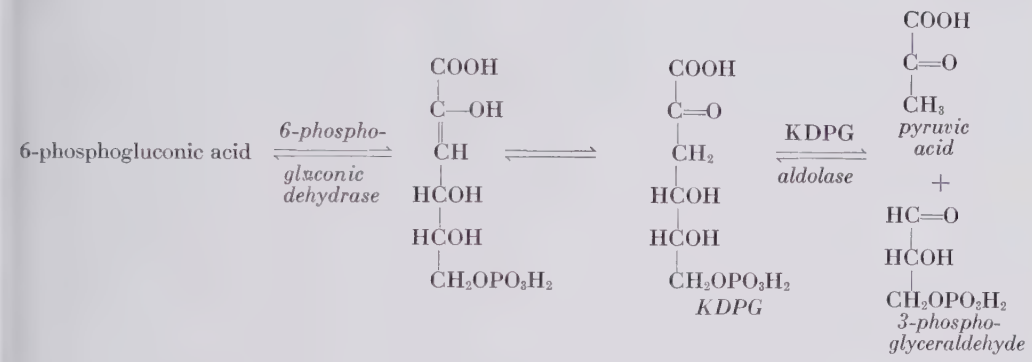


pathway in the same way that the splitting of fructose-1,6-diphosphate identifies the Embden-Meyerhof scheme (see **26** below). The 3-phosphoglyceraldehyde may then be converted by pyruvic acid by way of the reactions of pathway 2. The energy yield is one mole of ATP for each mole of glucose utilized.

Pathway 4: The Pentose Shunt.[62] This pathway differs from the other three in that the carbon skeleton of glucose is split at two different places in two separate reactions. First, 6-phosphogluconic acid is cleaved into a molecule of CO_2 and one of pentose by 6-phosphogluconic acid dehydrogenase, a TPN-specific enzyme. (See **27** below.)

The next step is the cleavage of the pentose molecule by the enzyme *transketolase* as shown in **28.** Its coenzyme diphosphothiamin (DPT) is firmly bound to the enzyme protein. The struc-

26.



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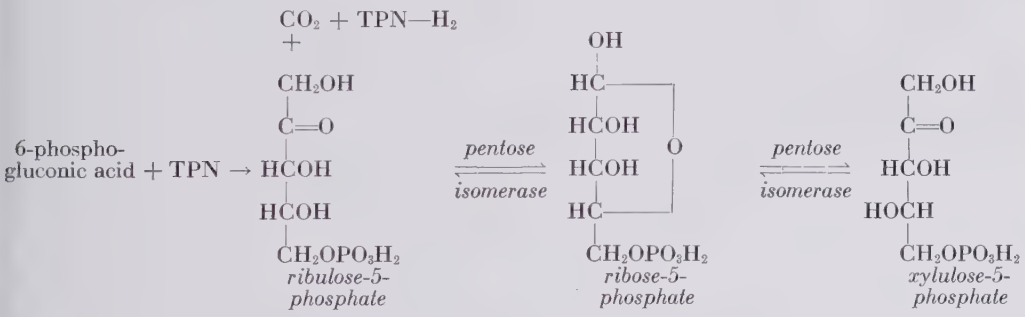

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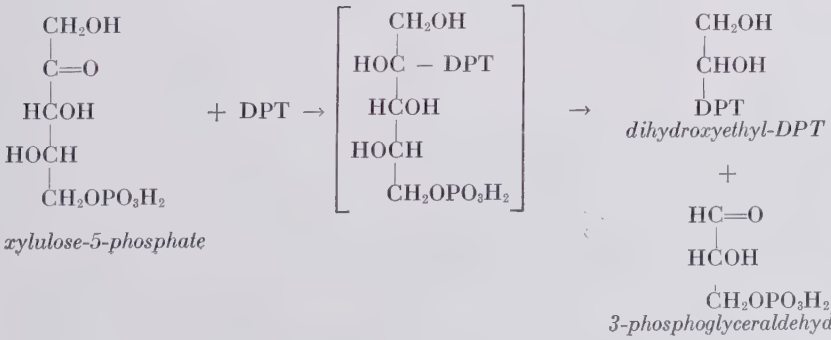


ture of DPT is given in **29**, which also shows the structure of *dihydroxyethyl-DPT*, the active 2-carbon intermediate formed by reaction between pentose and transketolase.[34] DPT forms analogous compounds in several other important reactions soon to be discussed.

At this point, the metabolism of pentose diverges in two directions, depending on the fate of the dihydroxyethyl-DPT. One pathway is the formation of lactate, acetate and ATP (**30**). Subtracting the mole of ATP used to form glucose-6-phosphate gives a net yield of two moles of ATP per mole of glucose, the same as in phosphorylating glycolysis. The formation of acetyl-coenzyme A and acetylphosphate will be discussed in detail in a later section.

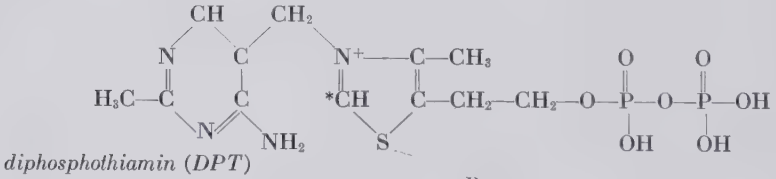
The other, more complicated pathway involves the conversion of three molecules of pentose to one molecule of 3-phosphoglyceraldehyde and two molecules of hexose. This *pentose cycle* is diagrammatically summarized on page 144. Since it affords a potential mechanism for the complete oxidation of glucose, it is shown as starting with hexose.

The first step is the oxidation of three hexose molecules (C_6) to pentose (C_5) as shown in **27**, page 141.

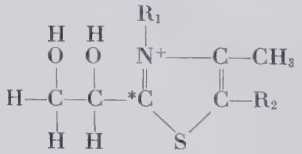
Second, two pentoses are cleaved by transketolase to dihydroxyethyl-DPT (C_2-DPT) and 3-phosphoglyceraldehyde (C_3) as described in **28**, page 141.

Third, in a reaction also catalyzed by transketolase, a molecule of dihydroxyethyl-DPT is transferred to the remain-

29.



diphosphothiamin (DPT)



dihydroxyethyl-DPT

30.

(a) xylulose-5-phosphate + DPT → dihydroxyethyl-DPT + 3-phosphoglyceraldehyde

(b) dihydroxyethyl-DPT + coenzyme A → acetyl-coenzyme A + DPT
acetyl-coenzyme A + H_3PO_4 → acetylphosphate + coenzyme A
acetylphosphate + ADP → ATP + acetic acid

(c) 3-phosphoglyceraldehyde + DPN + ADP + H_3PO_4 → 2-phosphoglyceric acid + ATP + DPN-H_2
2-phosphoglyceric acid + ADP → pyruvic acid + ATP
pyruvic acid + DPN-H_2 → lactic acid + DPN

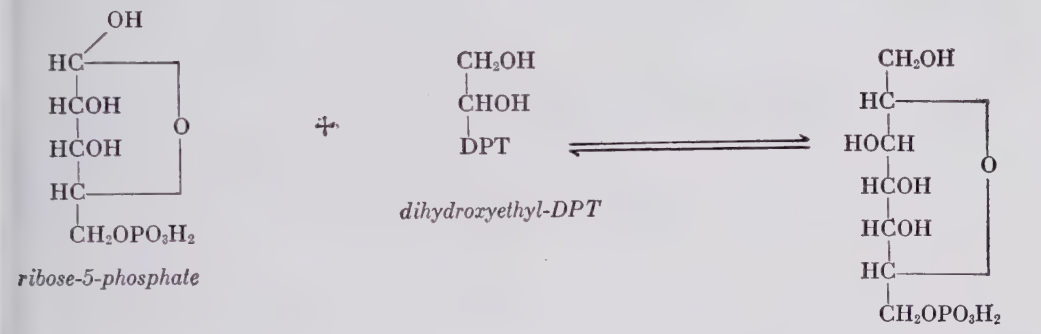
Net Reaction:
xylulose-5-phosphate + $2H_3PO_4$ + 3ADP → acetic acid + lactic acid + 3ATP

ing pentose to form the 7-carbon sugar, sedoheptulose (C_7), as shown in **31.**

Fourth, the enzyme *transaldolase* splits the sedoheptulose into the tetrose, *erythrose-4-phosphate* (C_4), and an active 3-carbon intermediate [C_3] (see **32**), which is immediately condensed with a molecule of 3-phosphoglyceraldehyde to form fructose-6-phosphate (see **33**).

Finally, the erythrose-4-phosphate and the remaining molecule of dihydroxyethyl-DPT are condensed by transaldolase

31.

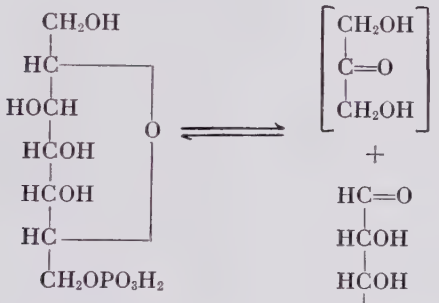


ribose-5-phosphate

dihydroxyethyl-DPT

sedoheptulose-7-phosphate

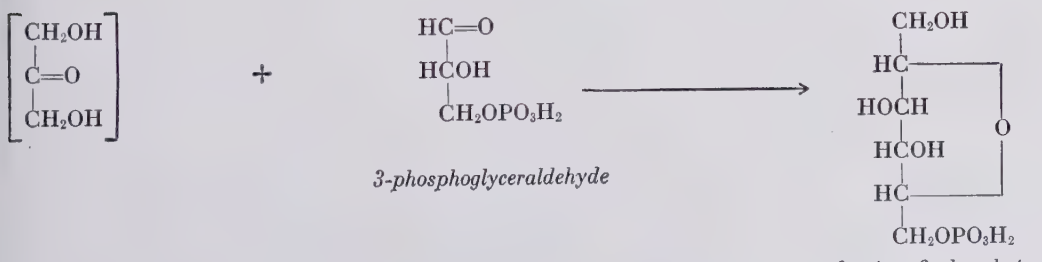
32.



sedoheptulose-7-phosphate

erythrose-4-phosphate

33.



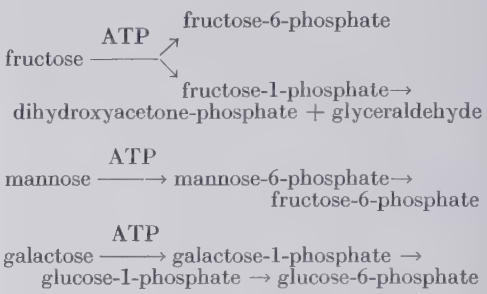
3-phosphoglyceraldehyde

fructose-6-phosphate

to produce a second mole of fructose-6-phosphate (see 34).

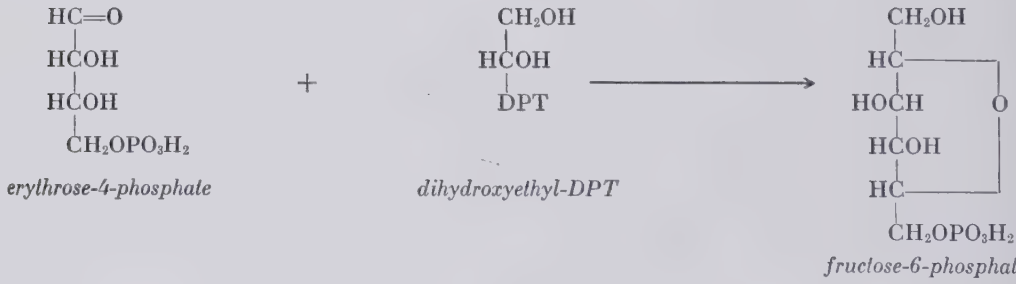
The net reaction is shown in **35.** The 3-phosphoglyceraldehyde may be metabolized anaerobically according to the fermentation pattern of the bacterium involved or may be completely oxidized by way of pyruvic acid and the Krebs cycle (see below). The fructose-6-phosphate may be aerobically repassed through the pentose cycle or anaerobically degraded by any of the mechanisms already discussed. The energy yield will depend upon the precise fate of the hexose and triose produced by the pentose cycle.

Breakdown of Other Hexoses and Pentoses. The many monosaccharides occurring in nature in either the free state or in oligosaccharides and polysaccharides are metabolized by conversion to intermediates in one or more of the pathways of glucose breakdown. Some typical conversions are:



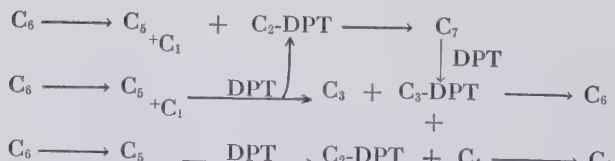
Relative Importance of Different Pathways of Glucose Metabolism.[11] Considerable efforts have been made to determine the relative importance of the different pathways of glucose breakdown in specific organisms. Of various methods used to estimate the amount of glucose broken down by each pathway, perhaps the most successful has been the

34.



35.

The Pentose Cycle



Net reaction:

$3C_6 \longrightarrow 3C_1 + C_3$ (*3-phosphoglyceraldehyde*) $+ 2C_6$ (*fructose-6-phosphate*)

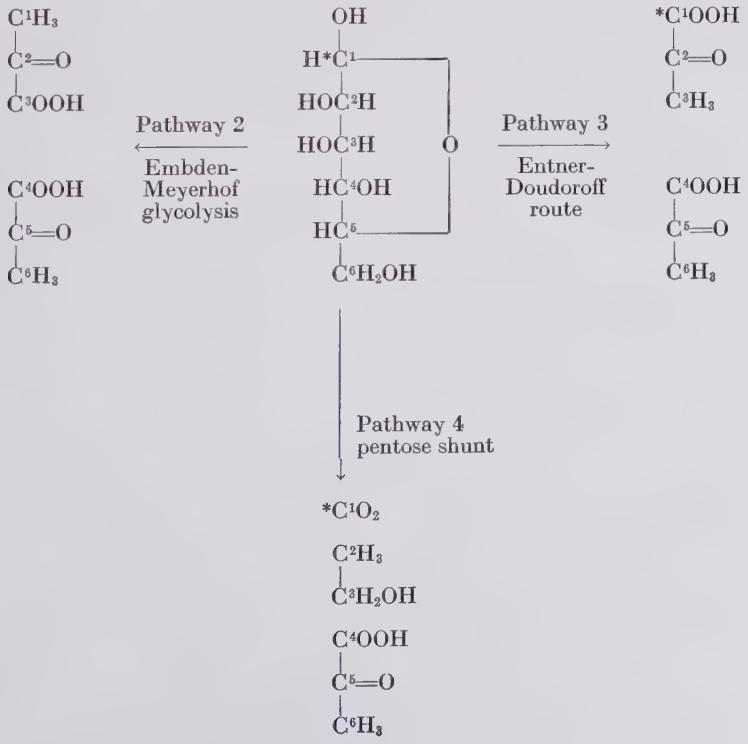
use of glucose labeled with the radioactive isotope of carbon, C^{14}. A simple example will illustrate the method. If glucose labeled in carbon 1 with C^{14} is degraded by pathways 2, 3, and 4, pyruvic acid may be a common product. However, note that the pyruvic acid formed by Embden-Meyerhof glycolysis contains the isotope in the methyl group of one of each two molecules formed, that the pyruvic acid produced by the Entner-Doudoroff route contains C^{14} in the carboxyl group of one molecule, and that the pyruvic acid formed by way of the pentose shunt contains none at all, because all the C^{14} was released as CO_2 (see **36**). Although the situation is by no means simple, quantitative studies of isotope distribution in the end products of glucose breakdown allow a reasonably reliable estimate of the relative importance of the various pathways. Some representative results are given in the accompanying table.[11]

Pathways of Glucose Breakdown

ORGANISM	PER CENT GLUCOSE DEGRADED BY		
	GLYCOLYSIS	PENTOSE SHUNT	ENTNER-DOUDOROFF PATHWAY
Lactobacillus casei	100		
Saccharomyces cerevisiae	88	12	
Escherichia coli	72	28	
Bacillus subtilis	59	41	
Leuconostoc mesenteroides		100	
Pseudomonas aeruginosa		29	71
Pseudomonas saccharophila			100

36.

Distribution of Carbon 1 of Glucose in the End Products of Different Pathways of Breakdown



In organisms exhibiting more than one mode of degradation (probably the major class), the exact relative proportions are dependent on many environmental conditions.

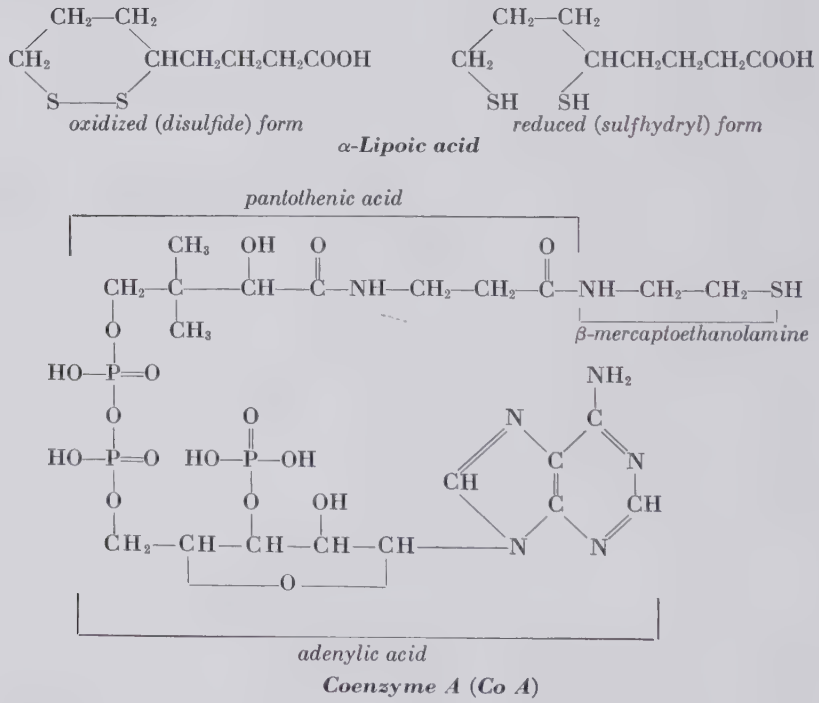
BREAKDOWN OF PYRUVIC ACID

Bacteria dissimilate pyruvic acid by what appear to be many different mechanisms into the variety of end products which are characteristic of bacterial fermentations. However, recent research has revealed common patterns in many apparently different modes of pyruvate breakdown which are probably of as regular and widespread occurrence as the Embden-Meyerhof glycolysis.

Coenzymes of Pyruvic Acid Metabolism. Three coenzymes are especially concerned in the metabolism of pyruvic acid: DPT, α-lipoic acid, and coenzyme A (Co A). Their constitution is indicated in the structural formulas in **29** and **37.**

Diphosphothiamin[34] is the coenzyme of the enzyme which catalyzes the decarboxylation of pyruvic acid to acetaldehyde. α-Hydroxyethyl-DPT is the primary product of pyruvate metabolism (see **38**). It may be decomposed to yield free acetaldehyde or it may be further metabolized.

37.



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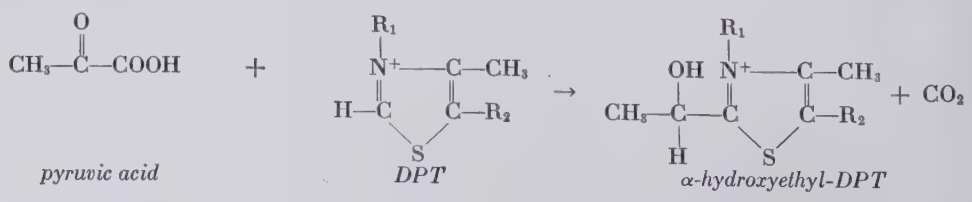


Other α-keto acids, such as α-ketoglutaric acid, are usually decarboxylated by similar DPT-enzymes.

α-Lipoic acid[51] is a reversibly oxidized and reduced coenzyme which is the hydrogen acceptor in the oxidation of the acetaldehyde produced by decarboxylation of pyruvic acid. (See **39.**)

α-Lipoic acid is also a growth factor for both bacteria and protozoa. It has been reported that DPT and lipoic acid occur in bacteria in combined form as *lipothiamide.*

Coenzyme A,[27] the acetylation coenzyme, is a complex structure composed of one molecule each of the growth factor pantothenic acid, adenylic acid, β-mercaptoethanolamine, and two molecules of phosphoric acid. It reacts with acetyl-α-lipoic acid to form acetyl-Co A. (See **40.**) The reduced form of α-lipoic acid released in the reaction is reoxidized by a DPN-enzyme.

Reactions of Pyruvic Acid. (1) ***Reduction.*** In Embden-Meyerhof glycolysis, L(+)-lactic acid is formed from pyruvic acid by a DPN-specific lactic dehydrogenase. Other lactic dehydrogenases are found in organisms not containing the Embden-Meyerhof system. *Leuc. mesenteroides* has a DPN-dehydrogenase which forms a D (−) isomer of lactic acid from pyruvic acid. Some lactic dehydrogenases, such as that of *L. delbruekii,* contain flavin nucleotides as coenzymes.

Pyruvic acid is also reduced to the amino acid alanine in reactions of transamination (see below).

(2) ***Nonoxidative decarboxylation and aldehyde transfer.*** These reactions have the same general mechanism and are catalyzed by DPT-containing enzymes. The first common step, the formation of α-hydroxyethyl-DPT, has already been described.

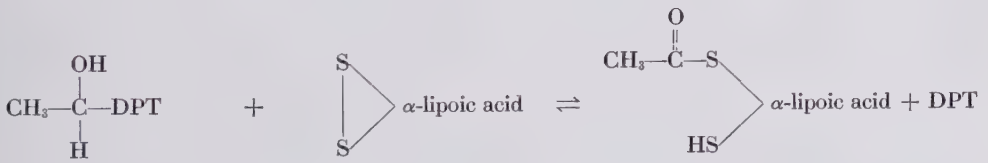
(A) *Direct Decarboxylation.* Yeast and many bacteria contain an enzyme, the classical pyruvic carboxylase of Neuberg, which directly decarboxylates pyruvic acid to free acetaldehyde and CO_2.

pyruvic acid + DPT-enzyme →
α-hydroxyethyl-DPT-enzyme + CO_2
α-hydroxyethyl-DPT-enzyme →
acetaldehyde + DPT-enzyme

The acetaldehyde may be reduced to ethanol, oxidized to acetic acid, or condensed with α-hydroxyethyl-DPT to form acetoin (see below).

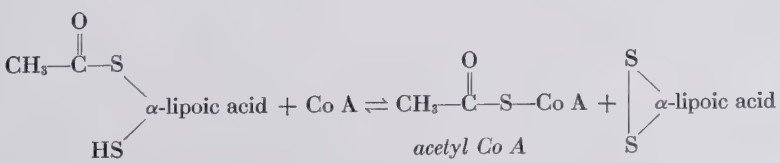
(B) *Transfer to Acetaldehyde.* Yeast, *E. coli,* and other organisms may trans-

39.



α-hydroxyethyl-DPT

40.



acetyl Co A

fer the α-hydroxyethyl group formed from pyruvic acid decarboxylation to a molecule of free acetaldehyde to form acetoin. (See **41.**) Acetoin may be reduced to 2,3-butanediol in some bacterial fermentations.

(C) *Transfer to Pyruvic Acid.* Another molecule of pyruvic acid may also accept the α-hydroxyethyl group to form acetoin by way of α-acetolactic acid (see **42**).

(3) ***Oxidative decarboxylation.*** The first step in both oxidative and nonoxidative decarboxylation of pyruvic acid is the formation of an acetaldehyde-DPT-enzyme complex. In the oxidative process, this intermediate is then transformed to a highly reactive form of acetic acid, acetyl-coenzyme A.

(a)

acetaldehyde-DPT + α-lipoic acid →
acetyl-α-lipoic acid-H_2 + DPT

(b)

acetyl-α-lipoic acid-H_2 + Co A $\overset{\text{transacetylase}}{\rightleftharpoons}$ acetyl-Co A + α-lipoic acid-H_2

The energy made available by the oxidation of pyruvic acid to acetic acid is conserved in the form of acetyl-α-lipoic acid which, like acetyl-Co A, is a high-energy form of acetate. Both are examples of the C–S type of high energy bond (see Respiration).

The reduced lipoic acid is reoxidized by DPN (as already discussed). The DPN may then in turn be oxidized either aerobically or anaerobically.

Acetyl-Co A may also be formed from acetic acid. Two different modes of acetate activation are operative in bacteria.

(a)

ATP + acetic acid $\overset{\text{acetokinase}}{\rightleftharpoons}$ acetylphosphate + ADP

acetyl-phosphate + Co A $\overset{\text{phosphotransacetylase}}{\rightleftharpoons}$ acetyl-Co A + inorganic orthophosphate

(b)

ATP + acetic acid → adenyl-acetate + inorganic pyrophosphate

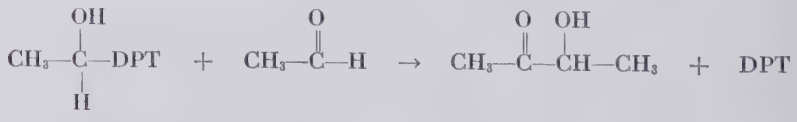
adenyl-acetate + Co A → acetyl-Co A + adenosine monophosphate

In these reactions the energy of ATP is used to form an intermediate high-energy form of acetate which then reacts with Co A to form acetyl-Co A.

(4) ***Reactions of acetyl-coenzyme A.*** (A) *Generation of High-Energy Phosphate Bonds.* The energy of Co A formed by any mechanism can be used to generate ATP from ADP and inorganic phosphate by reversing the reactions just given for the first mechanism for the conversion of acetic acid into coenzyme A (see **43** on opposite page).

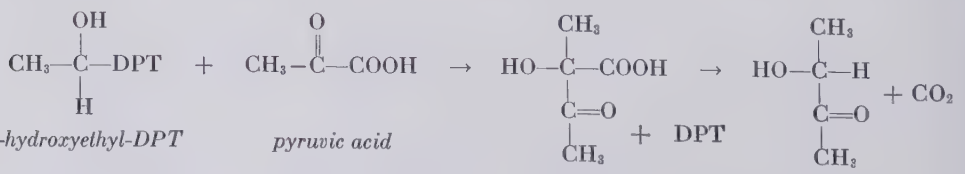
(B) *Donation of Acetyl Groups.* As important as the formation of high-

41.



α-hydroxyethyl-DPT *acetaldehyde* *acetoin*

42.



α-hydroxyethyl-DPT *pyruvic acid* *α-acetolactic acid* *acetoin*

energy phosphate is the role of acetyl-Co A as a form of "active acetate" used directly for synthetic purposes. This function is illustrated in the following two examples.

(a)

2 acetyl-Co A $\rightleftharpoons$ acetoacetyl-Co A + Co A

The formation of acetoacetyl-Co A is probably the first step in the formation of long-chain fatty acids and of the various end products of the butyric acid fermentations (see below).

(b)

acetyl-Co A + oxalacetic acid $\rightarrow$ citric acid + Co A

This reaction is the first step in the complete oxidation of carbohydrates by way of the Krebs cycle (see below).

(C) *Formation of Other Acyl-Co A Compounds.* Coenzyme A serves a function in the metabolism of other metabolically important acids similar to that just described for acetic acid. Acetyl-Co A may react with these acids to form the corresponding acyl-Co A derivatives in reactions catalyzed by the enzyme *Co A transphorase*. For example,

acetyl-Co A + succinic acid $\rightleftharpoons$ acetic acid + succinyl-Co A

The carbon skeletons of almost all cell constituents appear to be synthesized wholly or in part by addition of 2-carbon units to existing carbon chains by the acetylating action of acetyl-Co A. It is therefore apparent that the formation of acetyl-Co A represents a mechanism for the conservation and transfer of metabolic energy as important as the formation of ATP.

The chief reactions of pyruvic acid in bacteria, a few of which are still to be discussed in later sections of the chapter, are summarized in **44.**

43.

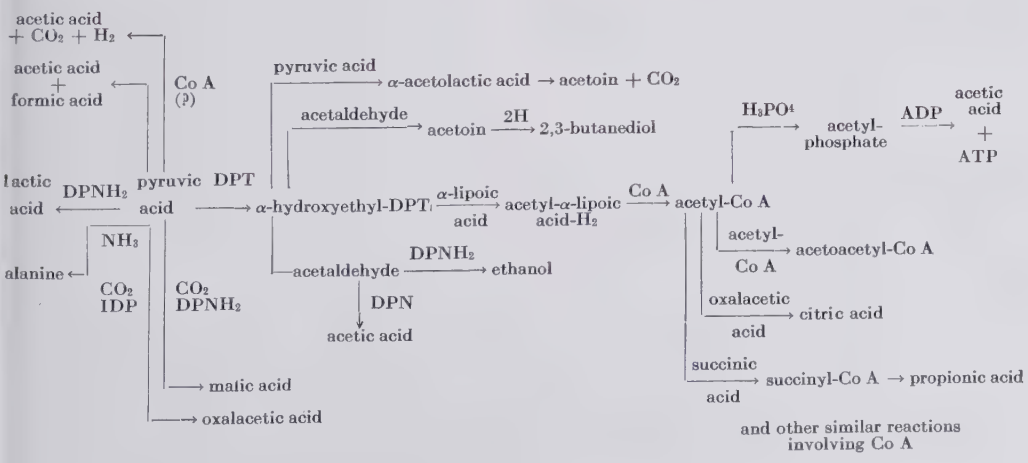
(a) $CH_3—C(=O)—S—$Co A + H_3PO_4 $\rightleftharpoons$ Co A + $CH_3—C(=O)—O—P(=O)(OH)—OH$

acetyl-Co A — *acetyl-phosphate*

(b) acetyl-phosphate + ADP $\rightarrow$ acetic acid + ATP

44.

Metabolism of Pyruvic Acid in Bacteria



THE TRICARBOXYLIC ACID CYCLE[32, 33, 35]

Aerobic bacteria can oxidize glucose completely to CO_2 and H_2O with molecular oxygen as the ultimate acceptor of electrons.

$$C_6H_{12}O_6 + 6\ O_2 \rightarrow 6\ CO_2 + 6\ H_2O$$

Glucose and other carbohydrates are converted to pyruvic acid by the different mechanisms already described. The pyruvic acid is then completely oxidized by the concerted action of a series of oxidizing and decarboxylating enzymes operating as a cyclic process. This is the *Krebs tricarboxylic acid cycle.*

Pyruvic acid enters the cycle by being converted into acetyl-Co A which in turn condenses with oxalacetic acid to form citric acid (see 46 on opposite page). The 6-carbon citric acid molecule is then decarboxylated and oxidized in a series of reactions to regenerate the 4-carbon oxalacetic acid molecule. Thus, the tricarboxylic and dicarboxylic acids of the cycle are not used up; *i.e.,* they act catalytically, but the pyruvic acid molecule is completely oxidized to CO_2 and H_2O. The reader can convince himself of this by summing up the oxygen used and the CO_2 and H_2O formed. (They are shown in bold face type in the diagram. Remember that H_2O is both formed and used up in the Krebs cycle.)

The enzymes involved in the Krebs cycle have been studied in great detail. The enzyme responsible for citric acid synthesis is known as the *condensing enzyme. Isocitric* and *malic dehydrogenases* are TPN- and DPN-proteins, respectively. The *α-ketoglutaric acid oxidase* system is very similar to the *pyruvic oxidate* (see 45 below). *Succinic dehydrogenase* is a cytochrome-linked dehydrogenase. *Oxalosuccinic decarboxylase* catalyzes the reversible nonoxidative decarboxylation of oxalosuccinic acid. Two *hydrases* are also active in the cycle, *aconitase* and *fumarase.*

Anything capable of generating acetyl-Co A can be oxidized by way of the Krebs cycle—pyruvic acid, acetic acid, fatty acids, etc. In addition, α-ketoglutaric and oxalacetic acids are in metabolic equilibrium with the key amino acids, glutamic and aspartic acids, respectively. Thus, the Krebs cycle is of great importance, not only as a mechanism of terminal respiration, but also as a common meeting ground for carbohydrate, fat and protein metabolism (see below).

Utilization of the Energy Liberated in the Tricarboxylic Acid Cycle. The most important product of the complete aerobic oxidation of carbohydrate is not CO_2 or H_2O, but energy. Approximately ten times as much energy of potential metabolic use is liberated in the complete oxidation of glucose to CO_2 and H_2O as in the glycolysis to lactic acid. In higher animals, this energy is efficiently conserved as high-energy phosphate. While there is a net synthesis of only two moles of ATP per mole of glucose converted to lactic acid, about thirty moles of ATP are formed for every mole of glucose oxidized by way of the Krebs cycle, and there is every reason to believe that the Krebs cycle oxidations are the chief energy sources for higher animals.

The few investigations which have been made of the coupling of aerobic oxidations with phosphorylation in bacteria all suggest that terminal oxidation of carbohydrate in bacteria is also accompanied by the formation of large amounts of energy-rich phosphate compounds. The familiar observation that a

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45.

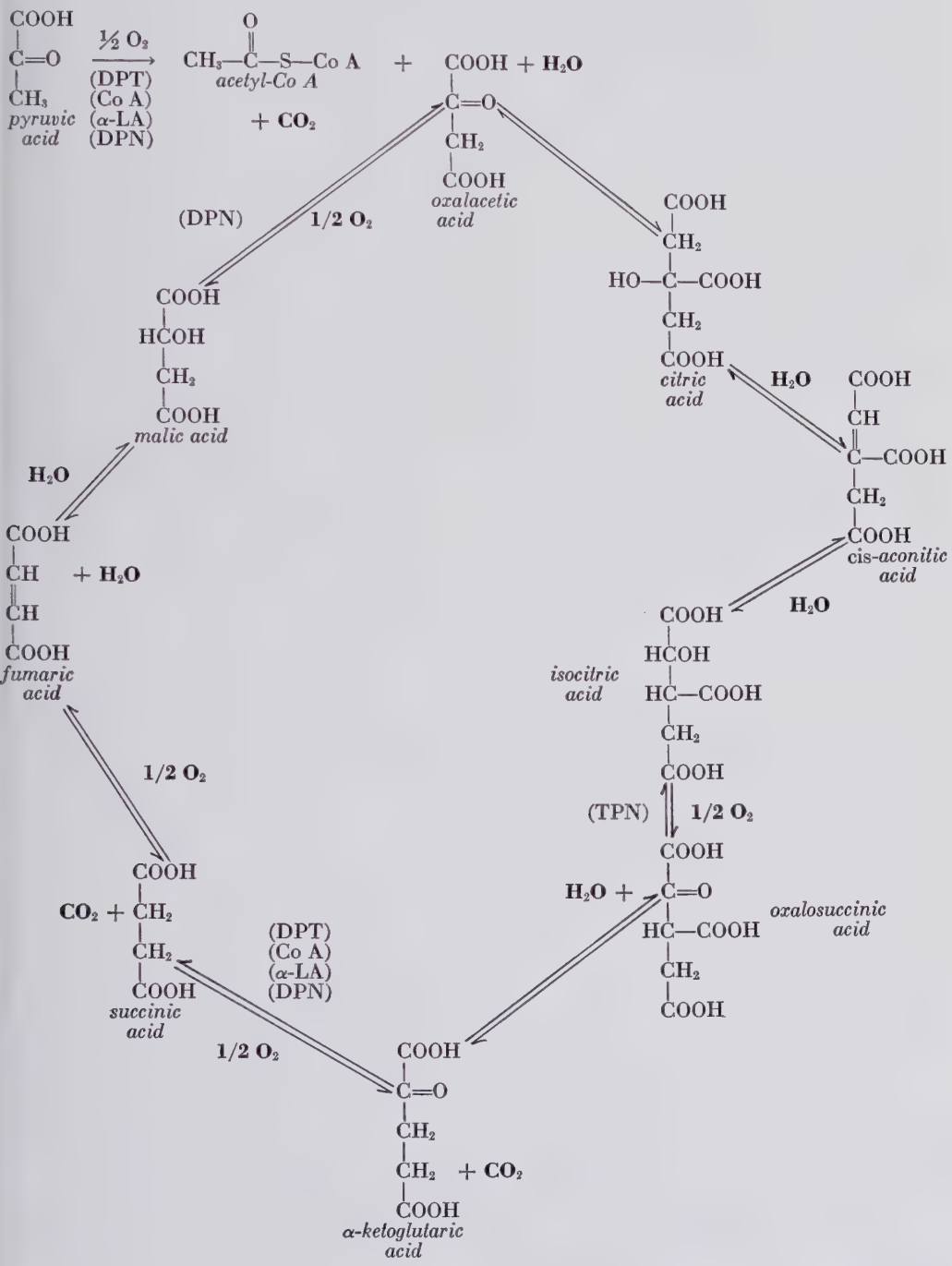
$$\alpha\text{-ketoglutaric acid} + \text{Co A} \xrightarrow[\alpha\text{-lipoic acid}]{\text{DPT}} \text{succinyl-Co A} + CO_2$$

$$\text{succinyl-Co A} + \text{guanosine diphosphate} + H_3PO_4 \rightarrow \text{succinic acid} + \text{Co A} + \text{guanosine triphosphate}$$

$$\text{guanosine triphosphate} + \text{ADP} \rightarrow \text{ATP} + \text{guanosine diphosphate}$$

46.

The Krebs Tricarboxylic Acid Cycle



Net reaction: $C_3H_4O_3 + 2\,\frac{1}{2}\,O_2 \rightarrow 3\,CO_2 + 2\,H_2O$
pyruvic acid

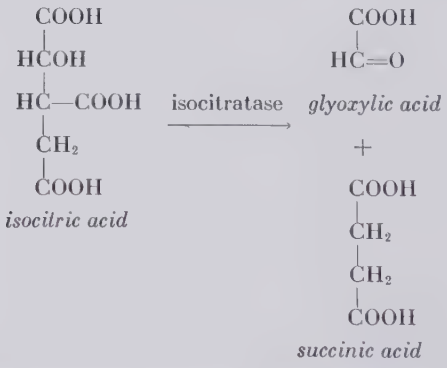
facultative anaerobe (such as *E. coli*), given the same amount of energy source to oxidize, will grow to a much higher cell density aerobically than anaerobically indicates that bacteria also derive much more energy from aerobic oxidations than from fermentations. However, bacteria appear to generate appreciably fewer energy-rich phosphate bonds per mole of glucose oxidized than do higher animals.

The Tricarboxylic Acid Cycle as a Source of Intermediates. The tricarboxylic acid cycle also serves an important synthetic function by providing carbon skeletons for the biosynthesis of many important cell constituents as, for example, α-ketoglutaric acid for the synthesis of glutamic acid. However, since each member of the cycle functions catalytically—one molecule is used up and one is formed for each turn of the cycle—this tapping of the tricarboxylic acid cycle for carbon skeletons will bring the operation of the cycle to an immediate halt if the acids withdrawn are not replaced.

Replacement occurs at the level of the 4-carbon dicarboxylic acids either by the fixation of carbon dioxide into 3-carbon acids (see below) or by a modification of the tricarboxylic acid cycle, the *glyoxylic acid cycle* (**49**).The two reactions of the glyoxylic acid cycle not shared with the conventional tricarboxylic acid cycle are catalyzed by the enzymes *isocitratase* and *malate synthetase* (see **47** and **48** below). Diagram **49** shows that for each turn of this modified cycle, two acetic acid molecules are converted into one molecule of succinic acid.

Since the tricarboxylic acid cycle generates energy and no intermediates, while the glyoxylic acid cycle generates intermediates but no energy, the bacterial cell must regulate the amount of acetyl-Co A fed into each cycle in order to meet changing metabolic demands. Regulation is achieved by control of both the activity and the synthesis of

47.



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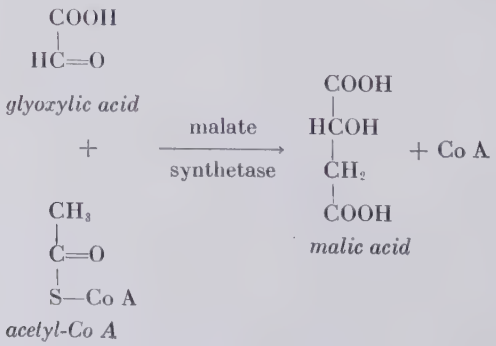
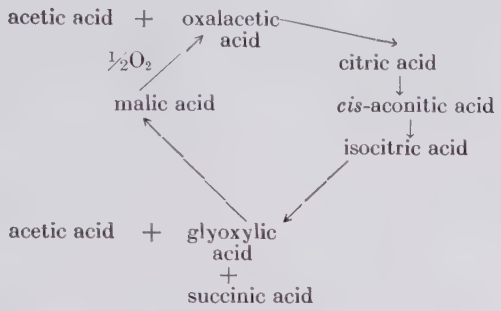


49.

The Glyoxylic Acid Cycle



Net reaction:

$$2 \text{ acetic acid} + \tfrac{1}{2}O_2 \rightarrow \text{succinic acid} + H_2O$$

isocitratase. This enzyme is strongly inhibited by succinic acid, one of the products of its action. Thus, it is active when the succinic acid concentration is low and inactive when it is high. Most bacteria synthesize some isocitratase under all conditions, but its production is inhibited when succinic acid is added to the growth medium and stimulated when acetic acid is the sole source of carbon. When acetic acid and other 2-carbon compounds are the sole sources of carbon, they must be assimilated by way of the glyoxylic acid cycle. Therefore, it must function at a high rate under these conditions.

FIXATION OF CARBON DIOXIDE

Almost all organisms possess, in widely varying degree, the ability to fix CO_2 into organic linkage. The general requirements for fixation are a source of energy and a source of electrons, because the reaction is an energy-requiring reduction. Living cells meet these requirements in many ways, but two main kinds of CO_2 fixation are clearly distinguished: *autotrophic fixation* and *heterotrophic fixation.*

Autotrophic and Heterotrophic Ways of Life. Introduction of these terms demands definition and explanation. All parasitic or saprophytic microorganisms derive their energy and most of their carbon from the oxidation of preformed organic compounds. In terms of the classification to be presented shortly, they are *chemoheterotrophs.* There is another large and widely distributed group of microorganisms of great biochemical interest and of critical importance in soil fertility and in the cycles of carbon, nitrogen, and sulfur in nature. These are the *autotrophic* bacteria. They obtain their energy from inorganic compounds or from light and their carbon from CO_2.

The word "autotroph" means simply "self-nourishing." In the sense used here, this refers to the lack of any *organic* nutritional requirement. Autotrophic bacteria obviously have the same general nutritional needs as heterotrophs: sources of energy, carbon, nitrogen, and essential inorganic ions.

It is appropriate at this point to inquire what is the essential difference between an autotroph and a heterotroph. Consider a representative of each metabolic type: the heterotroph *E. coli* grows in a medium containing only inorganic salts, ammonia, and an organic compound such as glucose; the autotroph *Thiobacillus thiooxidans* grows in a medium containing ammonia, elementary sulfur, and inorganic salts. The one absolute difference between the two is this: *E. coli* derives all of its energy from glucose oxidation and must have this oxidizable organic compound as an energy source in order to grow, while

Thiobacillus thiooxidans oxidizes sulfur for its energy and is completely independent of any organic energy supply. The second difference between *E. coli* and *Thiobacillus thiooxidans* is that the autotroph uses CO_2 as its sole carbon source and from CO_2 synthesizes all the organic carbon of its cell. Now, while *E. coli* can also utilize CO_2, it is insufficient as a sole carbon source and preformed organic carbon is also necessary for growth. Therefore, the second difference, the utilization of CO_2, is not absolute, but a matter of degree.

These differences in energy-yielding mechanisms and in the extent to which CO_2 serves as a carbon source are probably the only apparent differences between autotrophs and heterotrophs. In every other respect, the metabolism of autotrophic organisms appears to be just the same as that of heterotrophic organisms. Since autotrophs have no organic nutritional requirements, they must have great synthetic powers, greater than any other kind of organism. Thus, autotrophs have an exceedingly complex metabolism and cannot be the simple, primitive organisms they were once thought to be.

A useful classification of metabolic types of microorganisms based on energy and carbon requirements is as follows:

METABOLIC TYPE	ENERGY SOURCE	ORGANIC CARBON REQUIRED
Autotroph		
chemo-autotroph	inorganic compounds	no
photo-autotroph	light	no
Heterotroph		
chemoheterotroph	organic compounds	yes
photoheterotroph	light	yes

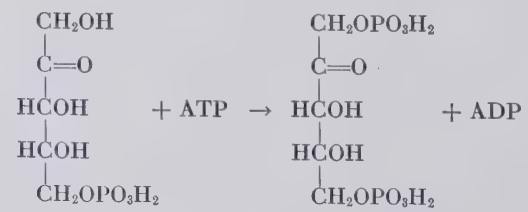
All types except the photoheterotrophs will be discussed in this section. Note that not all photosynthetic bacteria are also autotrophic. One point to be remembered in using this classification is that a given organism may, under different conditions, show different types of metabolism. Taking two examples, Hydrogenomonas can live as a chemoautotroph by oxidizing hydrogen or as a chemoheterotroph by oxidizing organic compounds. It is said to be a *facultative autotroph.* Such behavior is common; there are only a few *obligate autotrophs.* Similarly, *Rhodospirillum rubrum* grows anaerobically in the light as a photoheterotroph and aerobically in the dark as a chemoheterotroph.

Autotrophic Fixation of Carbon Dioxide.[5, 48] The cyclic mechanism whereby CO_2 is fixed into organic linkage by autotrophic organisms was discovered by Calvin and his associates in their studies on photosynthesis in unicellular green algae. However, the same cycle has also been shown to operate in the fixation of CO_2 by photo-autotrophic and chemo-autotrophic bacteria. Therefore, the original designation of the cycle as "the photosynthetic carbon reduction cycle" may be broadened to "the autotrophic carbon reduction cycle."

Only two enzymes are unique to this cycle. The rest also function in other areas of carbohydrate metabolism. One of the new enzymes is *phosphoribulokinase,* which catalyzes the reaction shown in **50.** The other enzyme, *carboxydismutase,* catalyzes the actual fixation reaction in which CO_2 is fixed to ribulose-1,5-diphosphate with the formation of two molecules of 3-phosphoglyceric acid. The exact reaction mechanism is still in doubt, but it is usually formulated as in **51** on the opposite page.

The remaining reactions of the autotrophic carbon reduction cycle are

50.

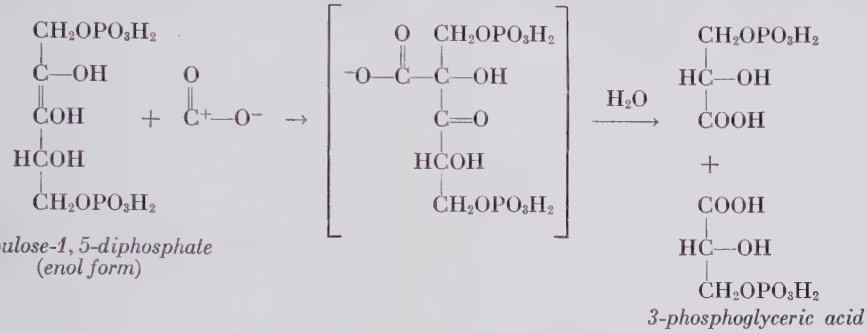


ribulose-5-phosphate *ribulose-1, 5-diphosphate*

concerned with (1) the reduction of 3-phosphoglyceric acid to 3-phosphoglyceraldehyde and (2) the regeneration of ribulose-5-phosphate. The reactions of the cycle are given in detail in **52.**

Reactions *c* through *g* also occur in Embden-Meyerhof glycolysis, while reactions *h* through *m* are also a portion of the pentose cycle. The autotrophic carbon dioxide reduction cycle may be summarized diagrammatically (**53**) in a

51.



ribulose-1, 5-diphosphate (enol form)

3-phosphoglyceric acid

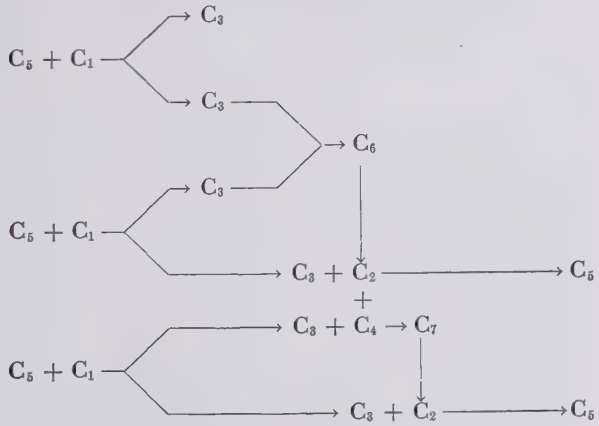
52.

(*a*) 3 ribulose-5-phosphate + 3 ATP → 3 ribulose-1,5-diphosphate + 3 ADP
(*b*) 3 ribulose-1,5-diphosphate + 3 CO_2 + 3 H_2O → 6 3-phosphoglyceric acid
(*c*) 6 3-phosphoglyceric acid + 6 ADP → 6 2,3-diphosphoglyceric acid
(*d*) 6 2,3-diphosphoglyceric acid + 6 (D or T)PN-H_2 →
6 3-phosphoglyceraldehyde + 6 (D or T)PN + 6 H_3PO_4
(*e*) 2 3-phosphoglyceraldehyde → 2 dihydroxyacetone-phosphate
(*f*) 3-phosphoglyceraldehyde + dihydroxyacetone phosphate → fructose-1,6-diphosphate
(*g*) fructose-1,6-diphosphate + H_2O → fructose-6-phosphate + H_3PO_4
(*h*) fructose-6-phosphate + DPT → erythrose-4-phosphate + dihydroxyethyl-DPT
(*i*) erythrose-4-phosphate + dihydroxyacetone-phosphate → sedoheptulose-1,7-diphosphate
(*j*) sedoheptulose-1,7-diphosphate + H_2O → sedoheptulose-7-phosphate + H_3PO_4
(*k*) sedoheptulose-7-phosphate + DPT → xylulose-5-phosphate + dihydroxyethyl-DPT
(*l*) xylulose-5-phosphate → ribulose-5-phosphate
(*m*) 2 3-phosphoglyceraldehyde + 2 dihydroxyethyl-DPT → 2 ribulose-5-phosphate + 2 DPT

Net reaction:

3 CO_2 + 9 ATP + 6 (D or T)PNH_2 + 5 H_2O → 3-phosphoglyceraldehyde +
9 ADP + 6 (D or T)PN + 8 H_3PO_4

53. **The Autotrophic Carbon Dioxide Reduction Cycle***



Net reaction:

3 C_1 → C_3
(*carbon dioxide*) (*3-phosphoglyceric acid*)

* Cf. The Pentose Cycle, **35**, on page 144.

form similar to that already used for the pentose cycle (**35**).

This cycle may be regarded as a modified pentose cycle in which occurs the unique reaction catalyzed by carboxydismutase. It has already been pointed out that CO_2 fixation is an energy-requiring reduction. It is now apparent that the energy is supplied by ATP and the reducing power by reduced pyridine nucleotide.

Carbon Dioxide Fixation by Chemo-autotrophic Bacteria.[36, 48] These organisms generate the ATP and reduced pyridine nucleotide required for CO_2 fixation by oxidizing inorganic compounds. They can grow in the dark in the presence of only CO_2, inorganic nitrogen, and essential inorganic ions. Although chemo-autotrophs have no organic requirements, they usually have very specific requirements as to energy source. Many can live as autotrophs only by oxidizing a single inorganic substrate. The principal chemo-autotrophic bacteria and their energy-yielding reactions are given in the table below:

Carbon Dioxide Fixation by Photo-autotrophic Bacteria.[56] Green plants generate their ATP and reduced pyridine nucleotide for CO_2 fixation from the energy of light in reactions occurring in the chloroplasts.

$$(a)\ ADP + P \xrightarrow{light} ATP$$

$$(b)\ ADP + P + TPN + H_2O \xrightarrow{light} ATP + TPN\text{-}H_2 + 1/2\ O_2$$

Reaction *a*, termed *cyclic photophosphorylation*, also occurs in photosynthetic bacteria, but reaction *b* does not. In *b*, TPN is reduced by way of the photochemical cleavage of H_2O, a reaction peculiar to green plant photosynthesis. In the photo-autotrophic green and purple bacteria, an oxidizable inorganic substrate is substituted for H_2O, and reduced pyridine nucleotide is generated enzymatically in the absence of a photochemical reaction. The most commonly employed substrate is H_2S:

$$H_2S + (D \text{ or } T)\ PN \rightarrow (D \text{ or } T)\ PN{-}H_2 + S$$

Heterotrophic Fixation of CO_2. It had been known for many years that CO_2 was involved in the metabolism of heterotrophic bacteria because it was frequently observed that many bacteria did not grow when all CO_2 was removed from the culture medium with CO_2-free air. However, proper interpretation of these observations was not made until 1935 when Wood and Werkman discovered that the heterotrophic propionic acid bacteria fix CO_2 into organic linkage, mainly in the carboxyl groups of the

Chemo-autotrophic Bacteria

GROUP	TYPICAL REPRESENTATIVE	ENERGY-YIELDING OXIDATION	AEROBIC OR ANAEROBIC	OBLIGATE AUTOTROPHE
Sulfur bacteria	Beggiatoa	$H_2S \rightarrow S$; $S \rightarrow SO_4^-$	aerobic	no
	Thiobacillus thioparus	$S_2O_3^- \rightarrow SO_4^- + S$	aerobic	yes
	Thiobacillus thiooxidans	$S \rightarrow SO_4^-$	aerobic	yes
	Thiobacillus denitrificans	$S \rightarrow SO_4^-$	anaerobic	yes
	Thiobacillus ferrooxidans	$Fe^{++} \rightarrow Fe^{+++}$	aerobic	yes
Nitrifying bacteria	Nitrosomonas, Nitrosococcus	$NH_3 \rightarrow NO_2^-$	aerobic	yes
	Nitrobacter	$NO_2^- \rightarrow NO_3^-$	aerobic	yes
Iron bacteria	Gallionella	$Fe^{++} \rightarrow Fe^{+++}$	aerobic	yes (?)
	Leptothrix	$Fe^{++} \rightarrow Fe^{+++}$	aerobic	no
Hydrogen bacteria	Hydrogenomonas	$H_2 \xrightarrow{O_2} H_2O$	aerobic	no
	Desulfovibrio desulfuricans	$H_2 \xrightarrow{SO_4^-} H_2S$	anaerobic	no
	Methanobacterium omelianskii	$H_2 \xrightarrow{CO_2} CH_4$	anaerobic	no

4-carbon dicarboxylic acids. Later investigations confirmed their original observation and demonstrated the occurrence of CO_2 fixation in other bacteria, fungi, protozoa, and higher animals.

CO_2 may be fixed by heterotrophs in $C_1 + C_1$, $C_1 + C_2$ and $C_1 + C_3$ additions. Of these, the $C_1 + C_3$ reactions are of greatest quantitative importance and will be the only ones discussed here.

Carboxylation of pyruvic acid to malic acid. The *malic enzyme* which catalyzes this reaction is widely distributed in bacteria.

$$\text{pyruvic acid} + CO_2 + \text{TPN—H}_2 \overset{Mn^{++}}{\rightleftharpoons} \text{malic acid} + \text{TPN}$$

Carboxylation of pyruvic acid to oxalacetic acid. This reaction occurs in at least two different ways. Phosphoenolpyruvate is an intermediate in both. In one reaction, its phosphate group is transferred to a nucleotide diphosphate, and in the other it is released as inorganic phosphate.

(*a*) phosphoenolpyruvic acid + CO_2 + IDP (or GDD or ADP) $\rightleftharpoons$ oxalacetic acid + ITP (or GTP or ATP)

(*b*) phosphoenolpyruvic acid + $CO_2 \rightarrow$ oxalacetic acid + P

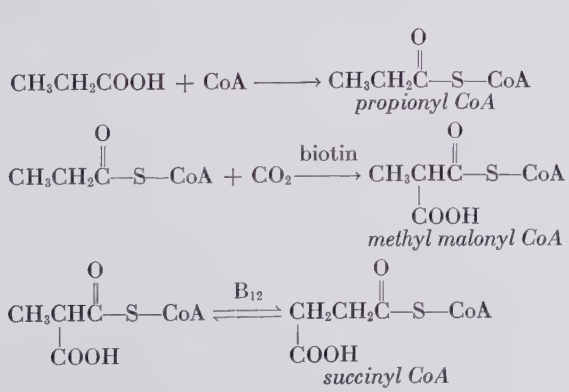
Carboxylation of propionic acid to succinic acid. This mode of CO_2 fixation is found chiefly in the propionic acid bacteria. It occurs as shown in **54** below:

The Importance of CO_2 Fixation. Organic carbon is constantly being converted into CO_2 by the respiratory activity of all living cells and by the breakdown of dead organic matter by saprophytic microorganisms. The CO_2 fixation of green plant photosynthesis is largely responsible for the return of this CO_2 to organic forms available to nonphotosynthetic organisms. Turning from the role of CO_2 in the economy of nature as a whole to its role in the economy of individual organisms, it is evident that, by definition, autotrophic organisms are completely dependent upon CO_2 as a carbon source. However, heterotrophic organisms cannot grow in the complete absence of CO_2, so CO_2 fixation must play a vital role in their metabolism as well. It is highly probable that at least one of the essential functions of CO_2 in heterotrophic economy is the replenishment of the 4-carbon members of the tricarboxylic acid cycle by means of the $C_1 + C_3$ additions just discussed. It is most difficult to estimate quantitatively how much of the carbon of a heterotrophic cell has been derived by fixation of CO_2, but the best estimates available are in the range of 15 to 30 per cent.

BACTERIAL FERMENTATIONS[62]

Bacteria anaerobically decompose, or ferment, carbohydrates to a relatively

54.



small number of 1-, 2-, 3-, and 4-carbon compounds. As indicated in **55**, fermentation end products are usually produced by the breakdown of glucose to pyruvic acid. The pyruvic acid then reacts in various ways, all of which have already been discussed, to form the typical end products of fermentation (indicated by bold face type in diagram below). In a general way, fermentation end products may be regarded as the reduction products of fermentation, with CO_2 as the oxidation product.

The chief metabolic value of the anaerobic breakdown of carbohydrate beyond the pyruvic acid stage lies in the extra energy made available, usually in the form of ATP. Bacterial fermentations are of practical importance because they produce products of industrial value (acetone, butanol, and so on) and because species differences in fermentation patterns are of great value in identifying and differentiating bacteria.

Most of the essential mechanisms involved in the metabolic transformations of bacterial fermentation have been considered in previous sections. The purpose of this section is to describe a few of the main types of fermentation patterns exhibited by important groups of bacteria.

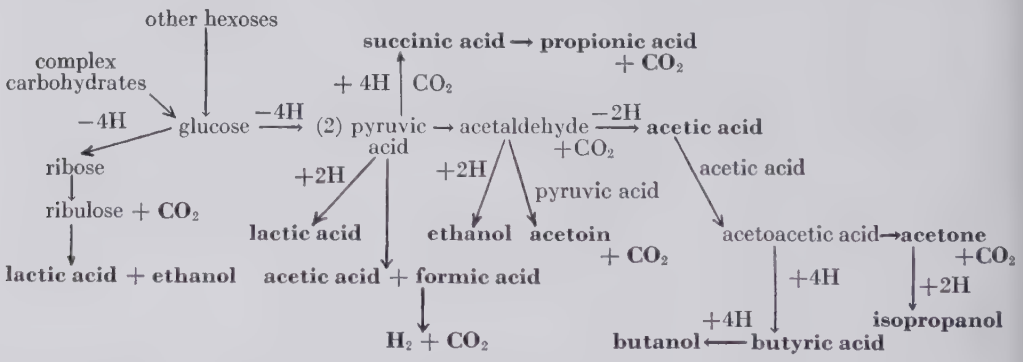
Alcoholic Fermentation. The oldest known type of fermentation is the production of ethanol from glucose.

$$\text{glucose} \rightarrow 2 \text{ ethanol} + 2\ CO_2$$

Yeasts carry out an almost pure alcoholic fermentation, but although some alcohol is produced by many bacteria, it is the major end product in only a relatively few species.

Glucose is converted by way of the Embden-Meyerhof pathway to pyruvic acid which is then decarboxylated to acetaldehyde by *pyruvic carboxylase* (**56**). The acetaldehyde is reduced to ethanol by *alcoholic dehydrogenase* (**57**).

55. **Bacterial Fermentation of Carbohydrates**



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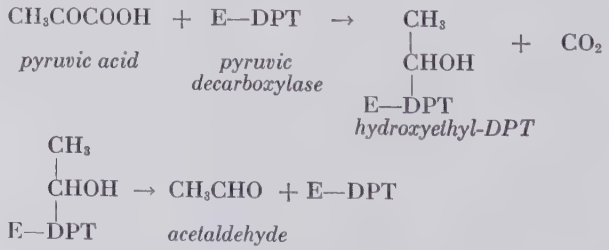
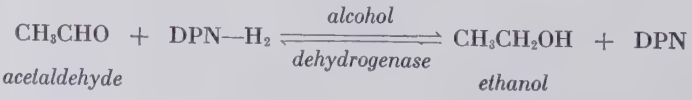

CH₃COCOOH + E—DPT → CH₃ | CHOH | E—DPT + CO₂
pyruvic acid
pyruvic decarboxylase
hydroxyethyl-DPT
CH₃ | CHOH | E—DPT → CH₃CHO + E—DPT
acetaldehyde

57.



Lactic Acid Fermentation. In a typical lactic fermentation, pyruvic acid produced from glucose by way of the Embden-Meyerhof scheme is reduced by DPN-H_2 formed in the oxidation of 3-phosphoglyceraldehyde.

pyruvic acid + $DPNH_2$ → lactic acid + DPN

A very large number of bacteria form some lactic acid, and it is the major fermentation end product of many (Lactobacillus, Streptococcus, Leuconostoc, Bacillus). If a given species, such as *L. casei*, produces only lactic acid from glucose by way of the Embden-Meyerhof pathway, it is said to possess a *homolactic* fermentation or to be a *homofermenter*. Others, such as *Lactobacillus brevis*, produce large quantities of lactic acid, but in addition form other end products such as acetic acid, ethanol, and CO_2. They are called *heterofermenters*, and the fermentation is termed *heterolactic* (see below). A single species may show both types of lactic fermentation, depending upon such environmental factors as pH and substrate.

In heterolactic fermentations it was formerly assumed that the end products are formed by way of the Embden-Meyerhof scheme with pyruvic acid as an intermediate. However, this is not always true. As has already been seen in previous sections, glucose may be broken down by way of pentose or the Entner-Doudoroff pathway to typical heterofermentative end products such as CO_2, ethanol, lactic acid, and acetic acid without the participation of phosphorylating glycolysis.

Propionic Acid Fermentation. The propionic acid bacteria are closely related to the lactobacilli and get their name from their characteristic production of propionic acid from glucose. The propionic acid bacteria also form acetic and succinic acids and CO_2 as typical fermentation end products.

Propionic acid is formed from pyruvic acid by the cyclic process shown in **58.** The cycle involves two novel reactions which merit attention (See **A** below).

First, CO_2 is transferred from methyl malonyl-Co A to pyruvic acid by a biotin-containing transcarboxylase without the formation of free CO_2. Biotin is also involved in other reactions of transcarboxylation.

Second, the conversion of succinyl-CoA to methyl malonyl-CoA requires the transformation of a straight-chain compound into a branched chain one. This is brought about by an isomerase containing a form of vitamin B_{12} (dimethyl-

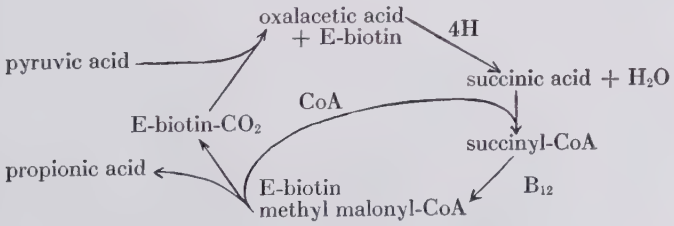
A.

methyl malonyl-CoA + E-biotin $\xrightarrow{\text{transcarboxylase}}$ propionyl-CoA + E-biotin-CO_2

E-biotin-CO_2 + pyruvic acid → oxalacetic acid + E-biotin

58.

Formation of Propionic Acid



Net reaction:

pyruvic acid + 4H → propionic acid + H_2O

benzimidazole-B_{12}) as its coenzyme (**59**).

Similar "chain-straightening" reactions also occur in the metabolism of amino acids.

Fermentations of the Coliform Group of Bacteria. Coliform bacteria ferment glucose to a variety of end products. *E. coli* produces lactic acid and ethanol by lactic and alcoholic fermentations. Lactic acid, acetic acid, and CO_2 are formed by the *dismutation* of two moles of pyruvic acid. By dismutation is meant the oxidation of one molecule and the reduction of the other. One molecule of pyruvic acid is oxidized to acetic acid and CO_2 as already described.

$$\text{pyruvic acid} + \text{DPN} \xrightarrow[\alpha\text{-lipoic acid}]{\text{DPT, Co A}} \text{acetic acid} + CO_2 + \text{DPN—}H_2$$

The other is reduced to lactic acid by lactic dehydrogenase and the DPN-H_2 produced in the oxidation of the first molecule of pyruvic acid.

$$\text{pyruvic acid} + \text{DPN—}H_2 \rightarrow \text{lactic acid} + \text{DPN}$$

The net reaction is:

$$2\ \text{pyruvic acid} \rightarrow \text{acetic acid} + \text{lactic acid} + CO_2$$

Acetic and formic acids are also formed by a reaction restricted to the enteric bacteria, the *phosphoroclastic* reaction. The over-all reaction is:

$$\text{pyruvic acid} + H_3PO_4 \rightleftharpoons \text{acetylphosphate} + \text{formic acid}$$

This reaction is CoA and DPT dependent:

$$\text{pyruvic acid} + \text{Co A} \overset{\text{DPT}}{\rightleftharpoons} \text{acetyl-Co A} + \text{formic acid}$$

$$\text{acetyl-Co A} + H_3PO_4 \rightleftharpoons \text{acetylphosphate} + \text{Co A}$$

ATP may be generated from acetylphosphate by reactions already described. If *E. coli* is grown in a medium with ammonium salts as the nitrogen source, formic acid is not further attacked, but if the medium contains certain amino acids, the enzyme *hydrogenlyase* is formed which breaks down formic acid to CO_2 and H_2.

$$HCOOH \rightleftharpoons CO_2 + H_2$$

The fermentation of glucose by *Aerobacter aerogenes* is similar to that of *E. coli* except that a considerable fraction of the fermented glucose is converted to acetoin. The production of acetoin is the basis for both the methyl red and the Voges-Proskauer tests for differentiation of *E. coli* from the aerogenes (cloaca) type of coliform bacilli. The Voges-Proskauer test is a color test for acetoin, while the formation of the neutral acetoin instead of acetic and formic acid makes the cloaca culture less acidic and thus methyl red–negative.

Butyl Alcohol Fermentations. Anaerobic organisms such as *Cl. butylicum*, *Cl. acetobutylicum*, *Cl. welchii*, and *Cl. kluyveri* possess glucose fermentations characterized by the accumulation of butanol, butyric acid, and related products. The key reaction in the formation of such compounds is the Co A-catalyzed formation of acetoacetic acid. From acetoacetic acid are formed the end prod-

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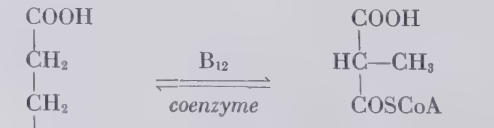
59.



ucts typical of the butyl alcohol fermentation. (See **60** below.)

The exact mechanisms by which some of these conversions take place and the intermediates involved are discussed in the section, Lipid Metabolism.

LIPID METABOLISM[2]

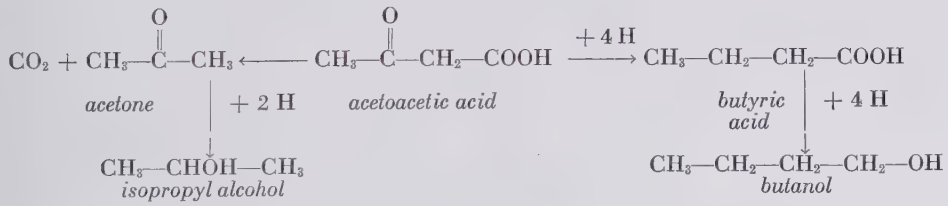
While microorganisms have played an important and sometimes dominant role in the development of basic concepts of carbohydrate metabolism, their contribution to basic concepts of lipid metabolism has been small, and most aspects of lipid metabolism are much less well understood in bacteria than in higher animals.

Bacteria contain appreciable quantities of a variety of lipids, the kind and amount frequently being highly dependent upon the exact conditions of culture. These lipids may be specifically incorporated into subcellular structures, such as cell walls and cytoplasmic membranes, or they may be stored as food reserves. Bacterial lipids differ from those of higher animals in the complete absence of cholesterol and other steroids and in the frequent presence of branched-chain fatty acids, hydroxy acids and glycolipids.[2]

Hydrolysis of Lipids.[31] *Neutral fats,* which consist of a molecule of glycerol esterified with three fatty acid molecules, are hydrolyzed by *lipases* (**61**).

Phosphatides, or *phospholipids,* contain a molecule of glycerol, two molecules of fatty acid, a molecule of phosphoric acid, and a nitrogen base which

60.



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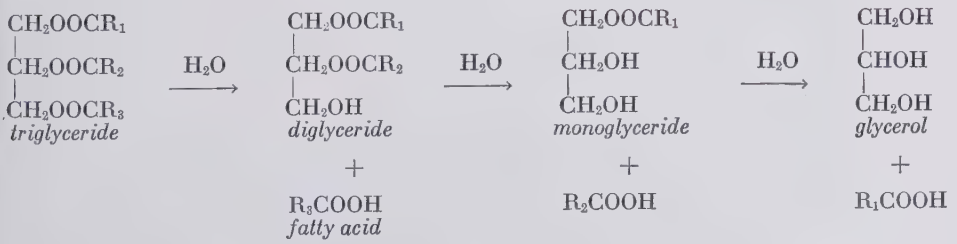


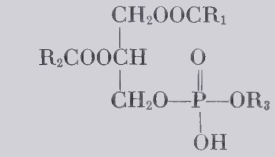
may be either choline, ethanolamine, or serine (**62**).

Choline-containing phosphatides are called *lecithins* (phosphatidylcholine). Those with ethanolamine and serine are called *cephalins* (more specifically, phosphatidylethanolamine and phosphatidylserine).

Lecithin may be used to illustrate the breakdown of phosphatides by bacteria (see **63**). *Phosphatidase A* (lecithinase A) hydrolyzes off one fatty acid molecule to produce *lysolecithin*, a potent hemolytic agent. This enzyme occurs in snake venom, bee stings, and scorpion venom and probably contributes to their toxic action. *Lysolecithinase* splits off another fatty acid to form *glycerylphosphorylcholine*. The same product is formed directly by the action of *phosphatidase B*. *Phosphatidase D* (lecithinase D) splits lecithin into a diglyceride and *phosphorylcholine*. This enzyme is identical with the α-toxin of *Clostridium welchii*.

Breakdown of Steroids.[57] Bacteria, actinomycetes, and fungi contain enzymes, usually inducible in nature,

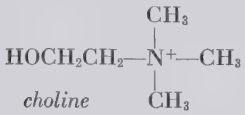
62.



basic structure of a phosphatide

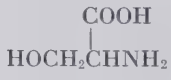
R_1 and R_2 = fatty acid radicals

R_3 = nitrogen base radical



choline

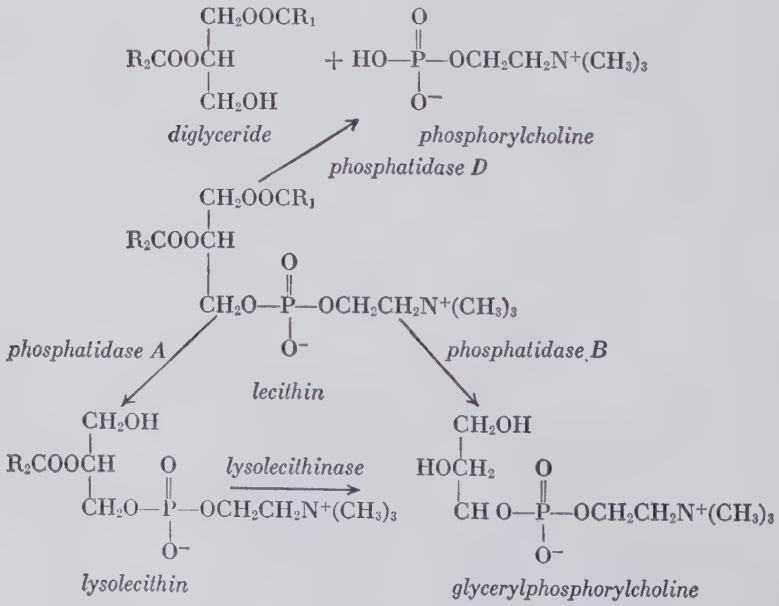
ethanolamine



serine

63.

Enzymic Hydrolysis of Lecithin



which catalyze oxidations and reductions of the side chains and nuclei of steroids. Some of these enzymes are of great importance in the industrial synthesis of steroid hormones.

Oxidation and Synthesis of Fatty Acids.[19] Microbial oxidation of fatty acids has been studied in detail in only a single species, *Clostridium kluyveri*. In this bacterium, fatty acids are oxidized by a mechanism closely similar to, but not identical with, the mitochondrial system for oxidation of fatty acids in higher animals. With butyric acid as an example, fatty acid oxidation proceeds as in **64**.

Reactions (2) through (5) are the same as in animal tissues, while reactions (1) and (6) are peculiar to bacteria.

Synthesis of fatty acids has also been studied in detail only in *Cl. kluyveri*. This organism converts ethanol to a mixture of butyric and caproic acids. Butyric acid is synthesized as shown in **65** on page 164.

In reactions (1) and (2) ethanol is oxidized to acetyl-CoA by way of acetaldehyde. The oxidation of acetaldehyde to acetyl-CoA is a reaction peculiar to *Cl. kluyveri* and involves the participation of diphosphothiamin. Reactions (3) through (6) are the reverse of corresponding reactions in the oxidation of butyric acid. Reaction (7) shows the different possible fates of butyrl CoA. In reaction (7b) butyryl CoA is converted into caproyl CoA by reactions similar to (3) through (6). This is assumed to be the mechanism whereby long-chain fatty acids are synthesized, by the addition of acetyl CoA to a shorter-chain acid.

Fatty acid synthesis and breakdown proceed by pathways that share many enzymes. Coenzyme A, in the form of acetyl-CoA and of higher acyl-CoA compounds, is of paramount importance in both processes.

Synthesis of Triglycerides and Phospholipids. Triglycerides are probably synthesized in bacteria by the esterification of glycerol with appropriate acyl-CoA compounds, but exact information is lacking.

Little more is known about phospholipid synthesis. In the single demonstrated bacterial synthesis of phospholipid the mechanism is not the same as in animal tissues (see **66** on page 164). The role of the cytidine nucleotides in phospholipid metabolism is similar to the role of the uridine nucleotides in carbohydrate metabolism (see Carbohydrate Metabolism).

64.

(1) $CH_3CH_2CH_2COOH + CH_3COSCoA \xrightleftharpoons{CoA-\ transphorase} CH_3CH_2CH_2COSCoA + CH_3COOH$
butyric acid *acetyl-CoA* *butyryl-CoA* *acetic acid*

(2) $CH_3CH_2CH_2COSCoA + \underset{\text{enzyme}}{\text{flavoprotein}} \xrightarrow[dehydrogenase]{acyl} \underset{crotonyl\text{-}CoA}{CH_3CH{=}CHCOSCoA} + \underset{\text{enzyme}}{\text{reduced flavoprotein}}$

(3) $CH_3CH{=}CHCOSCoA + H_2O \underset{hydrase}{\overset{enoyl}{\rightleftharpoons}} \underset{\beta\text{-}hydroxybutyrl\text{-}CoA}{CH_3CHOHCH_2COSCoA}$

(4) $CH_3CHOHCH_2COSCoA + DPN \underset{dehydrogenase}{\overset{\beta\text{-}hydroxyacyl}{\rightleftharpoons}} \underset{acetoacetyl\text{-}CoA}{CH_3COCH_2COSCoA} + DPN{-}H_2$

(5) $CH_3COCH_2COSCoA + CoASH \underset{thiolase}{\overset{\beta\text{-}ketoacyl}{\rightleftharpoons}} 2\ \underset{acetyl\text{-}CoA}{CH_3COSCoA}$

(6) $CH_3COSCoA + H_3PO_4 \underset{acetylphosphate}{\overset{phosphotransacetylase}{\rightleftharpoons}} CH_3COPO_3H_2 + CoASH$

65.

(1) $CH_3CH_2OH + DPN \underset{\text{dehydrogenase}}{\overset{\text{alcohol}}{\rightleftharpoons}} CH_3CHO + DPN{-}H_2$
ethanol *acetaldehyde*

(2) $CH_3CHO + DPN + CoASH \underset{\text{dehydrogenase}}{\overset{\text{aldehyde}}{\rightleftharpoons}} CH_3COSCoA + DPN{-}H_2$
acetyl-CoA

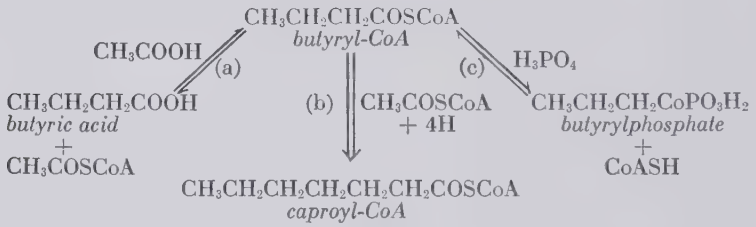
(3) $2\ CH_3COSCoA \underset{\text{thiolase}}{\overset{\beta\text{-ketoacyl}}{\rightleftharpoons}} CH_3COCH_2COSCoA + CoASH$
acetoacetyl-CoA

(4) $CH_3COCH_2COSCoA + DPN{-}H_2 \underset{\text{dehydrogenase}}{\overset{\beta\text{-hydroxyacyl}}{\rightleftharpoons}} CH_3CHOHCH_2COSCoA + DPN$
β-hydroxybutyrl-CoA

(5) $CH_3CHOHCH_2COSCoA \underset{\text{hydrase}}{\overset{\text{enoyl}}{\rightleftharpoons}} CH_3CH{=}CHCOSCoA + H_2O$
crotonyl-CoA

(6) $CH_3CH{=}CHCOSCoA + DPN{-}H_2 \rightleftharpoons CH_3CH_2CH_2COSCoA + DPN$
butyryl-CoA

(7)



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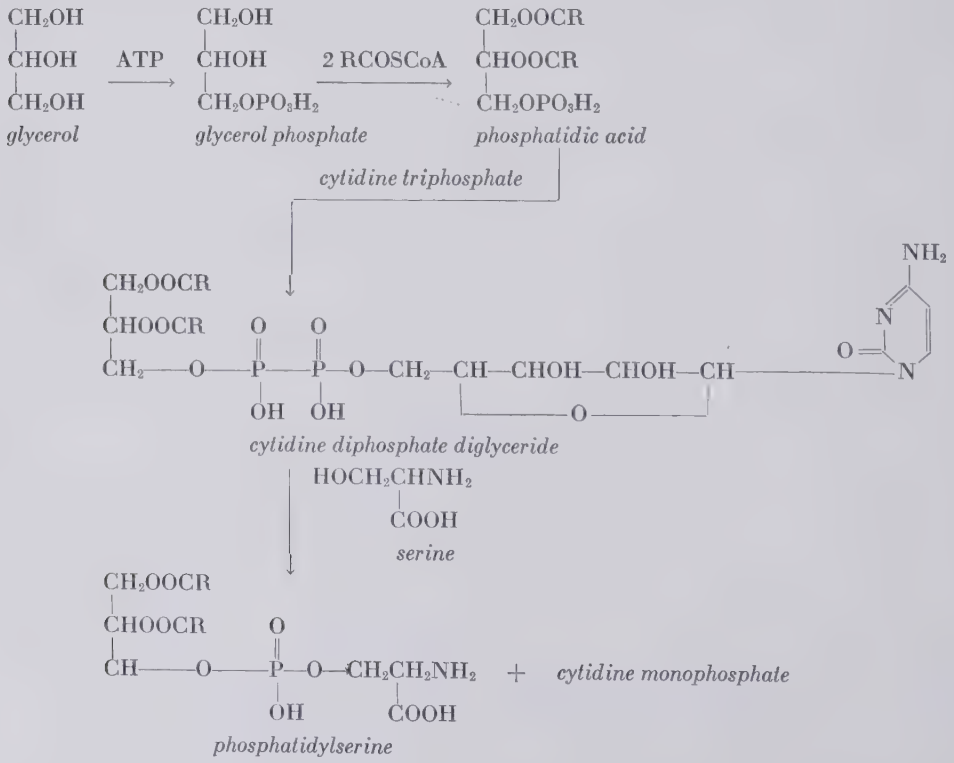
66. **Synthesis of Phosphatidylserine in *Escherichia Coli***



INORGANIC NITROGEN METABOLISM[36, 39, 40]

Nitrogen is an essential constituent of the last two great groups of metabolites to be considered, the amino acids and proteins and the nitrogen bases and nucleic acids. The metabolism of inorganic forms of nitrogen presents certain peculiar problems, stemming mainly from the unusual unreactivity of molecular nitrogen as compared with that of other elements near it in the periodic table—carbon, oxygen, silicon, phosphorus, and sulfur. These elements occur in large quantities in the rocks of the earth's crust, but nitrogen-containing minerals are much rarer, occurring almost exclusively in the South American nitrate deposits. Thus, the bulk of this vital element has remained as molecular nitrogen in the atmosphere.

Nitrate Metabolism. Green plants, the ultimate food source of all nonphotosynthetic organisms, can generally use the nitrates found in all soils and natural waters as their sole nitrogen sources. These nitrates are mainly obtained, not from nitrate deposits, which are very scarce, but from the breakdown of dead plant and animal matter in the soil.

Ammonia is produced in organic decompositions carried out by a wide variety of microorganisms.

$$\text{organic matter} \longrightarrow CO_2 + NH_3 + H_2O$$

However, the oxidation of ammonia to nitrate can be accomplished by only a few specialized obligately aerobic, obligately autotrophic soil microorganisms of universal distribution. The first step is the work of bacteria of the genera Nitrosomonas and Nitrosococcus:

$$2\ NH_3 + 3\ \ O_2 \rightarrow 2\ HNO_2 + 2\ H_2O$$

The nitrite is further oxidized to nitrate by Nitrobacter:

$$2\ HNO_2 + O_2 \rightarrow 2\ HNO_3$$

This process is called *nitrification* and the responsible organisms *nitrifying bacteria.*

The nitrogen cycle in nature may then be represented in a most simplified manner by:

$$NH_3 \rightleftarrows NO_3 \rightarrow \text{cell substance}$$

However, the cycle is not perfect and nitrates are constantly lost to biological use. In terrestrial nitrogen cycles, nitrates are constantly leached away by soil water and eventually lost in the ocean. In addition, some soil bacteria, the denitrifying bacteria, reduce nitrate to molecular nitrogen.

Nitrogen Fixation. If there were unlimited amounts of soil nitrate, this constant loss would be of no importance. However, since this is not so, it is fortunate that a number of microorganisms can reduce molecular nitrogen to ammonia and thus make up the deficit. Nitrogen fixation is a widespread but sporadically occurring metabolic process. While comparatively few microorganisms have been positively identified as nitrogen fixers, the organisms known to be capable of reducing molecular nitrogen include a wide variety of metabolic types as seen in the accompanying table.

Some Agents of Nitrogen Fixation

REPRESENTATIVE SPECIES	TYPE OF METABOLISM
Azotobacter chroococcum	aerobic, heterotrophic
Clostridium pasteurianum	anaerobic, heterotrophic
Nostoc (blue-green algae)	aerobic, photosynthetic
Rhodospirillum rubrum	anaerobic, photosynthetic
Rhizobium leguminosarum	aerobic, heterotrophic; fixation occurs only in association with leguminous plants

The first definitely identified product of the reduction of molecular nitrogen

is ammonia. Despite much experimental effort, practically nothing is known about the mechanism of this reduction and the possible intermediates involved.

The previous nitrogen cycle may now be enlarged to include nitrate loss and its replacement by nitrogen fixation, as shown in **67.**

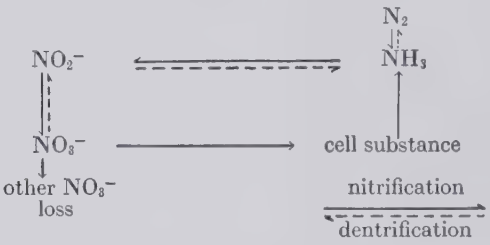
Although nitrogen, in many forms, is an essential constituent of all living cells, all these cells depend upon a very few different species of bacteria for the maintenance of a balanced nitrogen cycle in nature.

Primary Uptake of Ammonia into Organic Linkage. Since the nitrogen in most organic compounds of biological importance is at the state of oxidation of ammonia, it is not surprising that microorganisms generally introduce nitrogen into organic linkage in the form of ammonia.

Four main pathways for the primary uptake of ammonia are outlined in **68**, together with examples of important nitrogen-containing metabolites synthesized by each pathway. The detailed mechanisms of these reactions will be

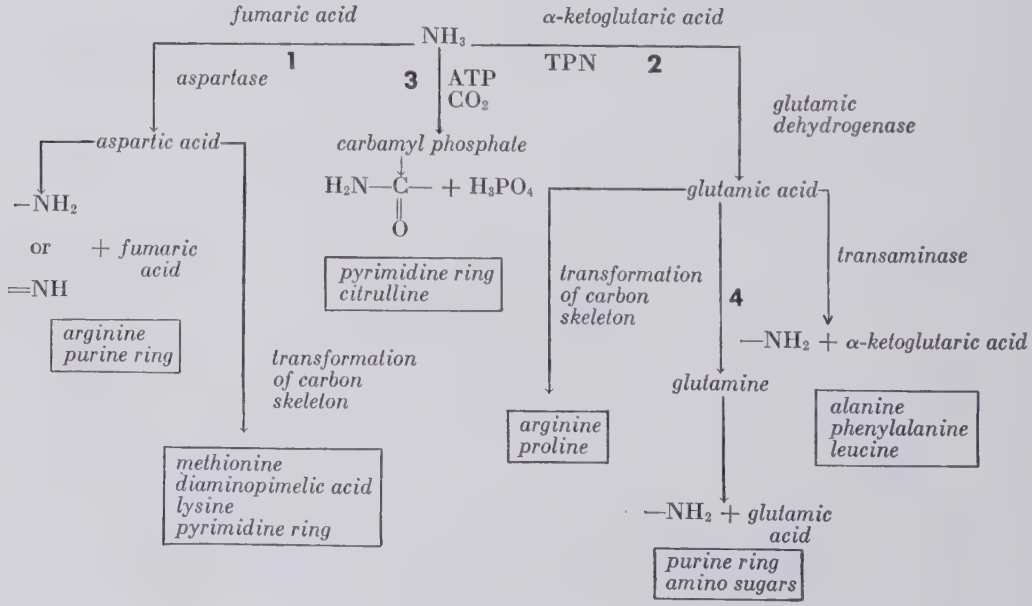
67.

The Nitrogen Cycle



68.

Primary Uptake of Ammonia into Organic Linkage



given at appropriate places in succeeding sections. It is sufficient to note at present that the primary products of ammonia uptake are: (1) aspartic acid, (2) glutamic acid, (3) carbamyl phosphate and (4) glutamine.

METABOLISM OF AMINO ACIDS AND PROTEINS

Amino acids occur in bacteria in the free state, in peptides, and in proteins. Most of them are found in proteins, but the small quantity of amino acids in the free state and in peptides is of great metabolic importance. Amino acids are not important energy sources for bacteria, except for the proteolytic anaerobes. Nevertheless, many bacteria degrade amino acids extensively as well as incorporate them into protein. Both the degradative and synthetic phases of amino acid metabolism are closely integrated with the metabolism of carbohydrates, particularly through the reactions of the Krebs cycle.

The general picture of amino acid metabolism is harder to visualize than that of carbohydrate metabolism because the twenty-odd amino acids occurring in proteins have widely different chemical structures and are, therefore, broken down and synthesized by different mechanisms. However, amino acids participate in many common reactions and are often metabolically related to each other. As these aspects of amino acid metabolism are better understood, the many seemingly diverse phases of amino acid metabolism assume a much greater measure of unity.

BREAKDOWN OF PROTEINS[53]

Intact protein molecules do not readily pass into the bacterial cell and must be hydrolyzed to smaller units by extracellular enzymes before they can be utilized as nitrogen sources. Relatively few bacteria possess such extracellular proteolytic enzymes. Bacteria are usually separated into fermentative and proteolytic types according to whether carbohydrate oxidation or proteolysis characterizes their biochemical activities. *Cl. sporogenes, Cl. histolyticum, Proteus vulgaris,* and *Ps. aeruginosa* are examples of actively proteolytic bacteria.

Enzymes responsible for protein breakdown are of two general types. *Proteinases* attack intact proteins and hydrolyze them mainly to peptides. They may be either intracellular or extracellular. *Peptidases* hydrolyze peptides to free amino acids. In bacteria, they appear to be chiefly intracellular. Only a few bacterial enzymes have been purified and studied in detail, but they appear to resemble closely the proteinases and peptidases of higher plants and animals. Bacterial enzymes attack a wide variety of proteins and peptides.

BREAKDOWN OF AMINO ACIDS[12, 20]

The ability to attack amino acids is much more widespread among bacteria than the ability to attack proteins. Bacteria may be divided into two groups on the basis of the variety of amino acids they are capable of breaking down. Gram-negative organisms such as *E. coli, Pr. vulgaris,* and *Ps. aeruginosa* attack almost all of the naturally occurring amino acids. Such organisms are also versatile synthesizers of amino acids, since they can grow on ammonia and a simple carbon source. In contrast, gram-positive organisms, such as the lactobacilli and the streptococci, have only a very limited ability to catabolize amino acids and also require a large number of amino

acids as growth factors. Thus there is a striking correlation between the ability to degrade amino acids and the ability to synthesize them. Bacteria, particularly gram-positive species, can accumulate high concentrations of free amino acids within their cell walls, much higher than the extracellular concentration of the amino acids in the medium. This is accomplished by means of an energy-requiring enzyme-like transport mechanism located at or near the cell surface.

Bacteria attack the amino acid molecule in two main ways: decarboxylation or deamination. The enzymes catalyzing both types are usually inducible; *i.e.*, they appear only when their substrates are present in the medium.

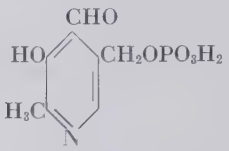
Decarboxylation of Amino Acids. Bacteria carry out a nonoxidative decarboxylation of amino acids to the corresponding amines, as shown by the general equation:

$$R{-}CHNH_2{-}COOH \rightarrow R{-}CH_2NH_2 + CO_2$$

Each decarboxylase is specific for a single amino acid, and the decarboxylation is complete and irreversible. Thus, an amino acid may be quantitatively determined by measuring the CO_2 liberated by its specific decarboxylase.

Enzymes decarboxylating diaminopimelic acid, lysine, arginine, ornithine, histidine, tyrosine, phenylalanine, aspartic acid, and glutamic acid have been described. The amino acid decarboxylases are widely distributed in many different bacteria, and one species may contain several of these enzymes.

With the possible exception of histidine decarboxylase, the amino acid decarboxylases have a common coenzyme, pyridoxal phosphate, which is a derivative of the growth factor pyridoxin or vitamin B_6.



pyridoxal phosphate

Pyridoxal phosphate is also a coenzyme for other enzymes important in amino acid metabolism (see below).

Deamination of Amino Acids. Bacteria deaminate amino acids in so many different ways that it is impossible to arrange the various mechanisms in an entirely orderly manner, but the following discussion attempts to bring together the different reactions of deamination into a few related groups.

Deamination of amino acids by the enteric bacteria. As a group, the enteric bacteria deaminate a large number of amino acids in several different ways. Reactions in *E. coli* have been studied with particular thoroughness.

Oxidative Deamination. Glutamic acid is broken down to ammonia and α-ketoglutaric acid by the glutamic acid dehydrogenase of *E. coli* as shown in **69** below. This reaction is readily reversible, and glutamic acid can be synthesized from ammonia and α-ketoglutaric acid. Since glutamic acid can transfer its amino group to many other keto acids by transamination (see below), fixation of ammonia nitrogen in glutamic acid

---

69.

$$\underset{\textit{glutamic acid}}{HOOC{-}CH_2{-}CH_2{-}\overset{\displaystyle NH_2}{\overset{|}{C}}H{-}COOH} + TPN \rightleftharpoons HOOC{-}CH_2{-}\underset{\textit{α-iminoglutaric acid}}{\overset{\displaystyle NH}{\overset{\|}{C}}{-}COOH} + TPN{-}H_2$$

$$\underset{\textit{α-ketoglutaric acid}}{HOOC{-}CH_2{-}CH_2{-}\overset{\displaystyle NH}{\overset{\|}{C}}{-}COOH} + H_2O \rightleftharpoons HOOC{-}CH_2{-}CH_2{-}\overset{\displaystyle O}{\overset{\|}{C}}{-}COOH + NH_3$$

by glutamic dehydrogenase is of primary importance in the synthesis of amino acids in general.

Many enteric bacteria (but not *E. coli*) contain amino acid oxidases which carry out the aerobic oxidative deamination of amino acids according to the general equation shown in **70** below.

The amino acid oxidases are usually nonspecific with respect to substrate; the oxidase of *Pr. vulgaris* attacks eleven different amino acids.

Nonoxidative Deamination. *E. coli* and related organisms contain enzymes catalyzing the deaminations shown in **71.** The coenzyme requirements of these deaminases are very complex. Pyridoxal phosphate is required for serine and cysteine deamination, while biotin, adenosine monophosphate, and a coenzyme of unknown chemical composition are concerned in the deamination of aspartic acid and serine.

The deamination of aspartic acid by aspartase is easily reversible and furnishes a second important pathway for the uptake of ammonia into amino acids, since aspartic acid, like glutamic acid, is very active in transamination reactions.

E. coli attacks tryptophan only in its side chain and leaves the aromatic rings intact in the form of indole (*cf.* the degradation of tryptophan by pseudomonads).[20] The reaction is shown in **72** below. Pyridoxal phosphate is the coenzyme for the reaction, and neither serine nor alanine is an intermediate.

Tryptophan Breakdown. In contrast to the limited attack made upon tryptophan by *E. coli,* most Pseudomonas spe-

70.

$$\underset{\textit{amino acid}}{R{-}CHNH_2{-}COOH} + \tfrac{1}{2}\,O_2 \rightarrow \underset{\textit{keto acid}}{R{-}CO{-}COOH} + NH_3$$

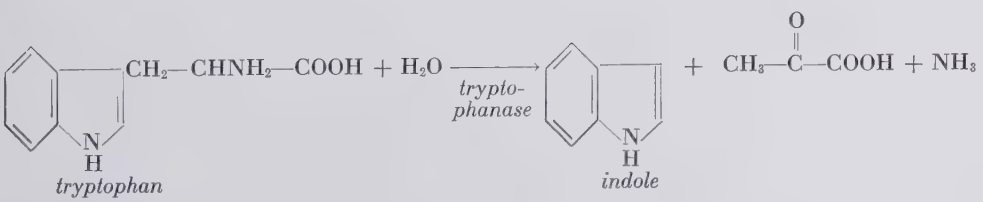
71.

$$\underset{\textit{aspartic acid}}{HOOC{-}CH_2{-}CHNH_2{-}COOH} \xrightleftharpoons{\text{aspartase}} \underset{\textit{fumaric acid}}{HOOC{-}CH{=}CH{-}COOH} + NH_3$$

$$\underset{\textit{serine}}{CH_2OH{-}CHNH_2{-}COOH} \xrightarrow[\text{dehydrase}]{\text{serine}} \underset{\textit{pyruvic acid}}{CH_3{-}CO{-}COOH} + NH_3$$

$$\underset{\textit{cysteine}}{CH_2SH{-}CHNH_2{-}COOH} + H_2O \xrightarrow[\text{desulfurase}]{\text{cysteine}} \underset{\textit{pyruvic acid}}{CH_3{-}CO{-}COOH} + NH_3 + H_2S$$

72.



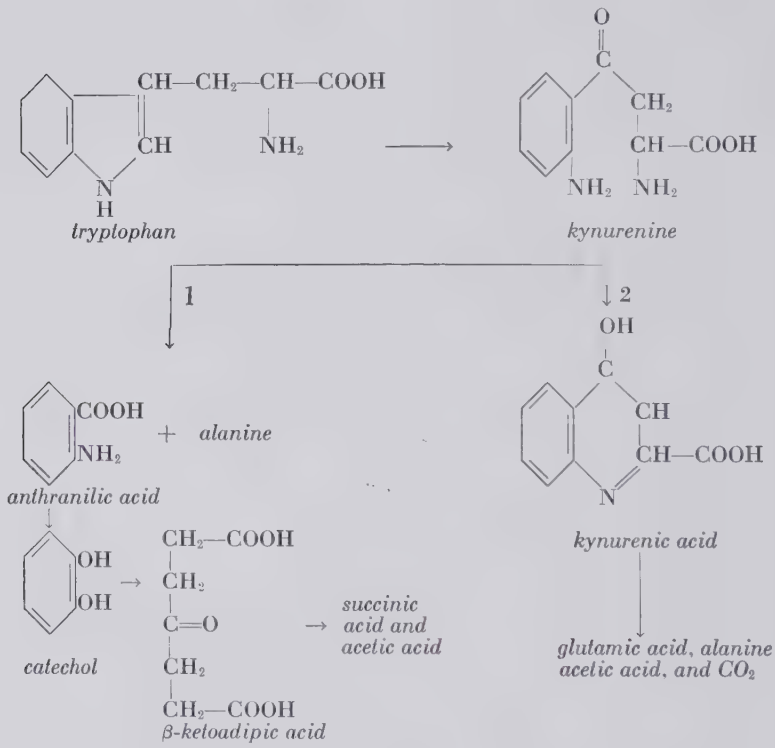
cies attack both the aromatic rings and side chains of the amino acid (see diagram **73**). All species tested form kynurenine, but at this point, depending upon the species, tryptophan breakdown proceeds (1) by a pathway leading to the destruction of both rings of the tryptophan molecule or (2) by a pathway leading to kynurenic acid and other products.

In higher animals and in the mold Neurospora, tryptophan is converted by way of kynurenine to the growth factor nicotinic acid (**74**). However, tryptophan is not a precursor of nicotinic acid in bacteria.

Metabolism of Amino Acids by Obligate Anaerobes. The proteolytic species of the genus Clostridium do not ferment carbohydrate and must obtain

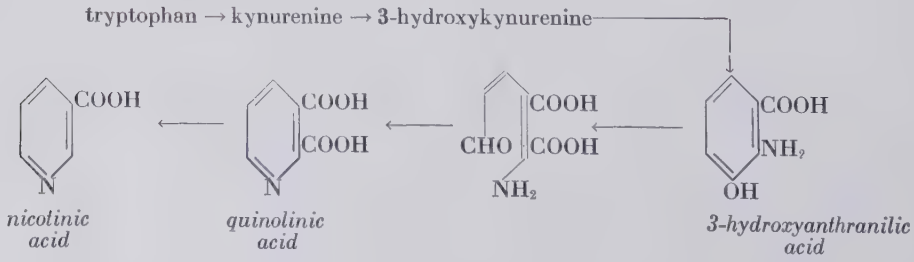
73.

Tryptophan Breakdown by Pseudomonads



74.

Tryptophan Breakdown by Neurospora



metabolic energy from the oxidation of amino acids. Since they are obligate anaerobes, the ultimate hydrogen acceptor is not molecular oxygen but the amino acids themselves or their breakdown products.

The clostridia have evolved two methods for the anaerobic oxidation of amino acids. In one, the Stickland reaction, one amino acid is oxidized and one is reduced. In the second, amino acids are decomposed with the liberation of molecular hydrogen.

The Stickland reaction. *Cl. sporogenes, Cl. botulinum,* and other (but not all) species perform reactions of the general type shown in **75.**

Amino acid (1) accepts hydrogen atoms from (2) and is reduced to a fatty acid while (2) is oxidized to a keto acid. The equation indicates only the over-all course of the reaction. It is likely that at least two enzymes, specific dehydrogenases for the reacting amino acids, and a hydrogen-carrying coenzyme, perhaps one of the pyridine nucleotides, are required for a Stickland reaction.

An example of a specific Stickland reaction is that between glycine and alanine (**76**). Amino acids participating in Stickland reactions fall into two groups, the hydrogen donors and the hydrogen acceptors. Any hydrogen donor will give a Stickland reaction with any hydrogen acceptor.

The coupled oxidation-reduction of amino acids in these reactions proceeds at a rate comparable to the aerobic oxidation of carbohydrate in fermenters, and the Stickland reactions appear entirely capable of serving as the chief energy source for the proteolytic clostridia. In at least one Stickland reaction, inorganic phosphorus is esterified and ATP is synthesized.

Decomposition of amino acids with liberation of hydrogen. Several clostridia which do not perform Stickland reactions decompose amino acids with liberation of NH_3, CO_2, and H_2. *Cl. tetanomorphum* breaks down glutamic acid according to equation **77** below.

The mechanism of such an "amino acid fermentation" is unknown, but the oxidation is obviously balanced by liberation of molecular H_2.

SYNTHESIS OF AMINO ACIDS[12, 21]

Heterotrophic bacteria synthesize amino acids from inorganic nitrogen, usually ammonia, and from simple organic carbon compounds such as acetic and pyruvic acids supplied in the medium or derived from the breakdown of other substances, usually carbohydrates. The great variation in structure among the twenty-odd amino acids usually found in proteins means that amino acids are made by many different independent synthetic routes. However, several reactions or groups of reactions are of general importance in amino acid synthesis and will be considered before the formation of individual amino acids is discussed.

75.

$$R_1\text{—}CHNH_2\text{—}COOH + R_2\text{—}CHNH_2\text{—}COOH + H_2O \rightarrow R_1\text{—}CH_2\text{—}COOH + R_2\text{—}CO\text{—}COOH + 2\ NH_2$$

76.

$$CH_2NH_2\text{—}COOH + CH_3\text{—}CHNH_2\text{—}COOH + H_2O \rightarrow CH_3\text{—}COOH + CH_3\text{—}CO\text{—}COOH + 2\ NH_3$$

glycine *alanine* *acetic acid* *pyruvic acid*

77.

$$5\ HOOC\text{—}CH_2\text{—}CH_2\text{—}CHNH_2\text{—}COOH + 6\ H_2O \rightarrow 6\ CH_3\text{—}COOH + 2\ CH_3\text{—}CH_2\text{—}CH_2\text{—}COOH + 5\ CO_2 + 5\ NH_3 + H_2$$

glutamic acid *acetic acid* *butyric acid*

Uptake of Ammonia. The primary uptake of ammonia into organic linkage has already been discussed (see "Inorganic Nitrogen Metabolism"). The ability to convert ammonia nitrogen into amino acid nitrogen is a common property of heterotrophic bacteria. Ammonia is the main primary source of nitrogen for heterotrophic bacteria. It is true that some bacteria can utilize molecular nitrogen, but the first detectable product of nitrogen fixation is ammonia, and once ammonia is formed, the nitrogen metabolism of nitrogen-fixers is no longer unique. Also, some bacteria such as the streptococci and lactobacilli require large numbers of amino acids as growth factors, yet they can utilize ammonia for the synthesis of still other amino acids not required. Thus the ability to convert ammonia nitrogen into amino acid nitrogen is a common property of heterotrophic bacteria.

It is not yet entirely clear how ammonia is incorporated into amino acids. Mechanisms for the formation of glutamic and aspartic acids from ammonia and the corresponding keto acids have already been given. It is probable that a considerable fraction of the utilized ammonia is incorporated by way of glutamic acid and transamination reactions into many amino acids. However, this cannot be the sole route of ammonia uptake because not all amino acids enter into transamination reactions and because there is good evidence that the corresponding keto acids are not always the direct precursors of amino acids.

Transamination. Many amino acids undergo the following general reaction called *transamination.* (See **78.**) In transamination, the amino group of one amino acid is transferred to the α-position of an α-keto acid, thus synthesizing a new amino acid and a new keto acid. *Transaminases* are widely distributed in animals, higher plants, and bacteria.

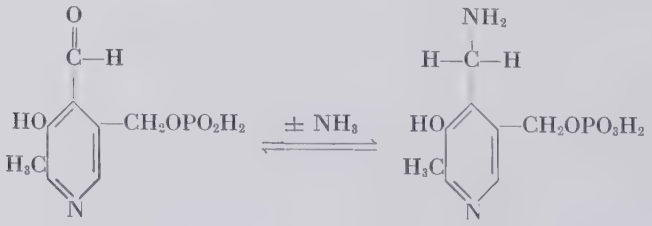
The transaminases are yet another group of amino acid enzymes for which pyridoxal phosphate is the coenzyme. Under appropriate conditions another member of the vitamin B_6 family, *pyridoxamine phosphate,* is also a coenzyme of transamination. (See **79.**) Snell has suggested that these coenzymes function in transaminase systems as amino group carriers. Thus, the reaction between glutamic and oxalacetic acids proceeds as shown in **80.**

Although many amino and keto acids

78.

$$R_1\text{—}CHNH_2\text{—}COOH + R_2\text{—}CO\text{—}COOH \rightleftharpoons R_1\text{—}CO\text{—}COOH + R_2\text{—}CHNH_2\text{—}COOH$$

79.



pyridoxal phosphate *pyridoxamine phosphate*

80.

enzyme-CHO + glutamic acid $\rightleftharpoons$ enzyme-NH_2 + α-ketoglutaric acid
(*pyridoxal form*) (*pyridoxamine form*)

enzyme-NH_2 + oxalacetic acid $\rightleftharpoons$ aspartic acid + enzyme-CHO

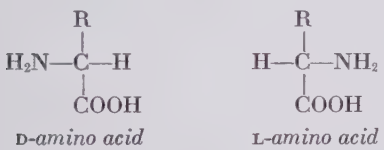
Net reaction:
glutamic acid + oxalacetic acid $\rightleftharpoons$ α-ketoglutaric acid + aspartic acid

participate in transamination reactions, glutamic and α-ketoglutaric acids have been a part of almost all transamination reactions so far described. These two acids may be thought of as an amino-group-carrying system,

$$\text{glutamic acid} \underset{\pm NH_3}{\rightleftharpoons} \alpha\text{-ketoglutaric acid}$$

which may either accept amino groups from other amino acids or donate them to other keto acids. Transaminations are generally reversible reactions, and an amino acid which can donate its amino group to α-ketoglutaric acid can also be synthesized by the reverse reaction from glutamic acid and its keto acid analogue. In bacteria, transamination of the keto acids corresponding to glutamic acid, aspartic acid, alanine, serine, phenylalanine, tyrosine, leucine, isoleucine, and valine appears to be an important synthetic reaction. There are many different transaminases, each with fairly definite substrate specificity.

Racemization and the Importance of D-Amino Acids. With the exception of glycine, each α-amino acid has an asymmetric carbon atom and therefore exists as two optical isomers, the D- and L-forms:



D-amino acid *L-amino acid*

By far the greater bulk of the amino acids of proteins are of the L- configuration, and it was once thought that only the L- form occurred in nature. The D-forms were often called "unnatural" amino acids. However it is now clear that D-amino acids are widely distributed in bacteria. They were first demonstrated in various polypeptides characteristic of the genus Bacillus: in the capsule of *B. anthracis* (D-glutamic acid) and in the antibiotics gramicidin and tyrocidine from *B. brevis* (D-leucine, D-valine, and D-phenylalanine). Penicillin contains D-valine and D-cycloserine is a derivative of D-serine. D-alanine and D-glutamic acid are essential structural components of a peptide apparently common to the cell walls of all bacteria. It appears that only the L-isomers of amino acids appear in bacterial protein while both D and L forms are found in peptides.

Although D-amino acids serve as growth factors for many bacteria, they are usually less effective than the L-amino acid or the α-keto acid, suggesting that they serve only for the formation of the corresponding L-amino acid. However, there is one clear-cut specific requirement for a D-amino acid. D-alanine, but not L-alanine, will replace pyridoxal in the growth of *Str. fecalis* R. In the absence of pyridoxal, added D-alanine is actively incorporated into cell material; and when pyridoxal is present, *Str. fecalis* synthesizes D-alanine.

These observations led to the discovery of a new type of enzyme, the *amino acid racemase.* The racemase catalyzes the racemization of optically active amino acids; *i.e.,* it converts either the D- or L- acid to the racemic DL- mixture. It was found that *Str. fecalis* contains an *alanine racemase,* which requires pyridoxal phosphate as its coenzyme, thus explaining why this organism cannot synthesize D-alanine when pyridoxal is omitted from the growth medium.

The presence of D-alanine and of alanine racemase has been demonstrated in a large number of bacteria, and it has been suggested that D-alanine is an essential metabolite for all bacteria because of its occurrence in the fabric of the cell wall. Racemases for glutamic acid, methionine, threonine, proline, and diaminopimelic acid have also been found in a number of bacteria. Other enzymes also attack D-amino acids, such as the D-serine dehydrase of *E. coli.* There is also evidence that D-aspartic acid is an essential metabolite for several bacteria. All these findings suggest that, despite the quantitative preponderance of L-amino acids in proteins, both the D- and L-isomers are essential components of bacterial protoplasm, and that enzymes for the interconversion of the optical isomers are of general occurrence.

Synthesis of Individual Amino Acids. The rapid increase in our knowledge of the mode of synthesis of the amino acids makes it impossible to consider each amino acid separately. Instead, discus-

sion will be limited to a few amino acids, for which the most detailed information is available.

Sulfur amino acids and hydroxyamino acids. Of these groups of amino acids only the sulfur acids cysteine, cystine, and methionine and the hydroxy acids serine and threonine appear in proteins in appreciable quantities, but there are severarl related amino acids of importance as synthetic intermediates. The metabolism of the sulfur and hydroxyamino acids is closely interwoven because the carbon skeleton of several pairs of these compounds is the same (example: cysteine and serine) and because cystathionine, a common metabolic intermediate, is formed by the combination of a sulfur amino acid and a hydroxyamino acid (see **81**).

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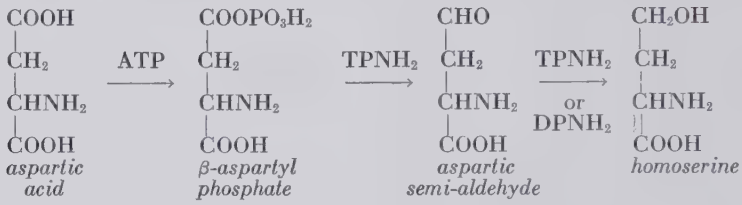
81. Synthesis of the Sulfur Amino Acids and the Hydroxyamino Acids

1.



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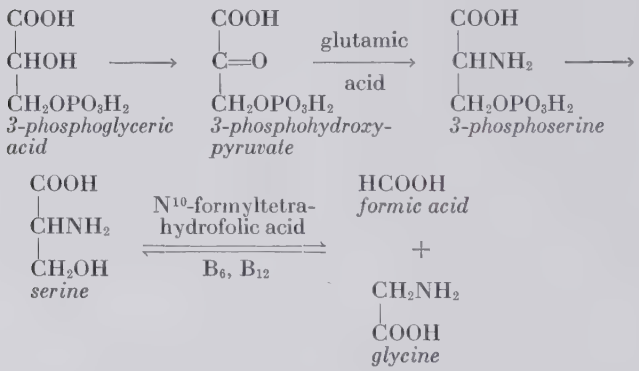

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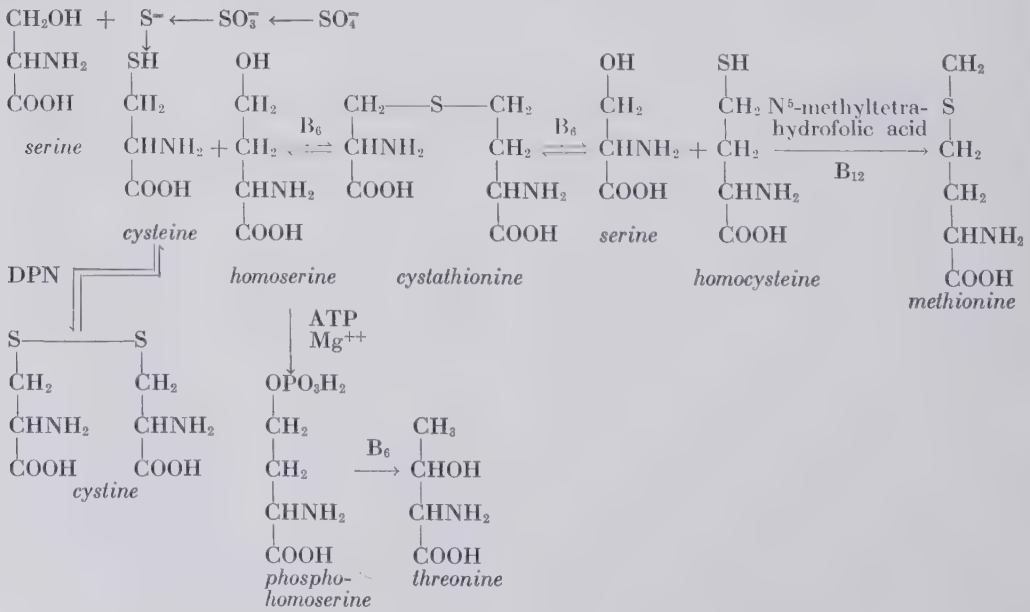


Since the carbon skeletons of cysteine, cystine, methionine, and threonine are derived from either serine or homoserine, the synthesis of these two amino acids will be considered first. Homoserine is made from aspartic acid as shown in **81, 1.** Serine is synthesized as shown in **81, 2,** and glycine is reversibly formed from serine. Many bacteria contain enzymes which catalyze the reversible conversion of glycine and formic acid to serine (**81, 2**). However, it is not certain whether serine is a precursor of glycine or vice versa.

Diagram **81, 3** describes the biosynthesis of cysteine, cystine, methionine, and threonine. Bacteria utilize inorganic sulfate for amino acid synthesis by reducing it first to sulfite and then to sulfide. The first sulfur amino acid to appear is cysteine, which reacts with homoserine to form cystathionine. The cystathionine is then split so as to leave the S attached to the homoserine residue, thus forming homocysteine and serine. Finally, the homocysteine is methylated to methionine. Therefore, methionine S comes from cysteine, but the precursor of the rest of the molecule is homoserine. Threonine arises from the rearrangement of homoserine.

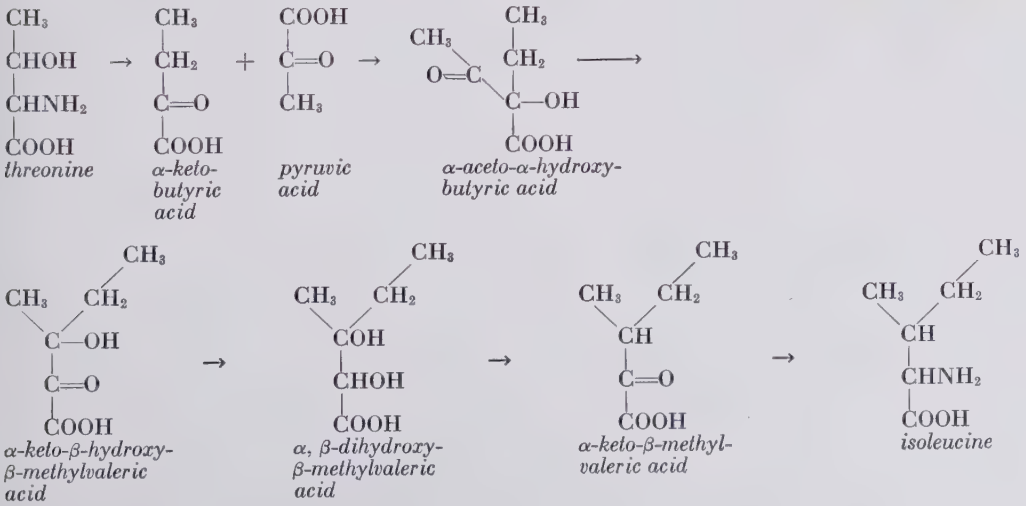
The syntheses of serine from glycine and of methionine from homocysteine are examples of a widespread type of reaction, commonly called "1-carbon transfer," which is also important in the synthesis of purines and pyrimidines. In these reactions a coenzyme form of the growth factor folic acid (see below) acts as an acceptor and donor of formyl groups much the same as Co A is known to accept and donate acetyl groups. Other coenzymes, vitamin B_{12} in particular, may also be necessary for transfer of 1-carbon units. Although formic acid can usually serve as the source of the 1-carbon unit, other substances may also donate 1-carbon fragments. They are usually compounds such as methionine and serine which are formed by 1-carbon transfer.

Isoleucine and valine. Isoleucine is synthesized by a relatively long series of reactions beginning with homoserine (see diagram **82**). Threonine is converted to α-ketobutyric acid which condenses with pyruvic acid to form eventually the dihydroxy analogue of isoleucine. The dihydroxy analogue is converted to the keto acid which accepts an amino group by way of transamination to form isoleucine.

Valine, which differs from isoleucine only in that it has two methyl groups instead of a methyl and an ethyl, is synthesized by an analogous pathway

82.

Synthesis of Isoleucine



Many of the steps in valine and isoleucine biosynthesis are catalyzed by the same enzymes.

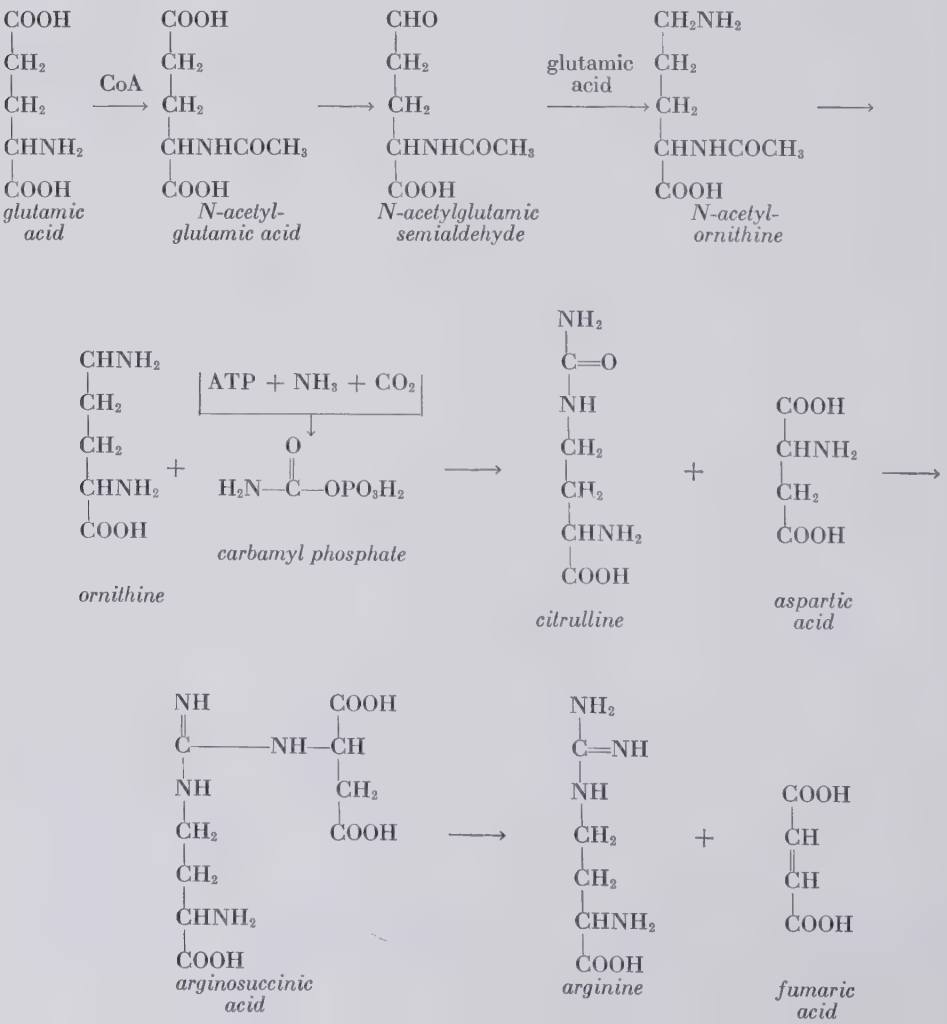
Arginine. Arginine is probably synthesized in all living things by the same general series of reactions, which in urea-producing animals has the additional importance of being a part of the Krebs-Henseleit urea cycle (see **83**). The carbon skeleton of arginine comes from glutamic acid by way of ornithine. Ornithine then reacts with carbamyl phosphate, formed from CO_2, NH_3, and ATP, to produce citrulline. Citrulline then combines with aspartic acid to form arginosuccinic acid which breaks down to arginine and fumaric acid.

In the synthesis of arginine, one begins to see how a bacterium growing on a simple carbohydrate and ammonia medium can synthesize its own amino acids. Starting with glucose and ammonia, the pathway of arginine synthesis could be as shown in **84** on the opposite page. Part of the metabolized glucose is oxidized to generate ATP which is used as a source of energy for the conversion of other glucose molecules into the intermediates needed for arginine synthesis.

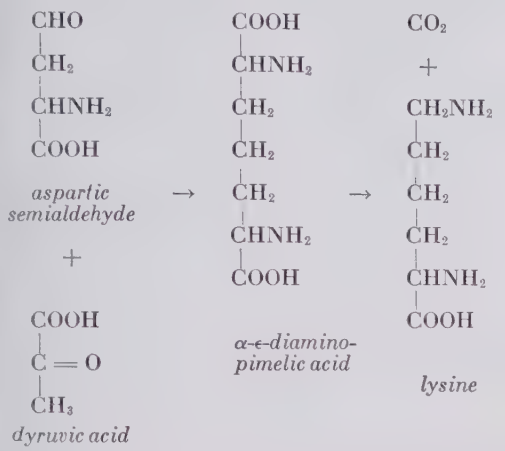
Diaminopimelic acid and lysine. α-ϵ-Diaminopimelic acid is a constituent of the cell wall peptide of many bacteria and is in addition the immediate precursor of lysine. They are formed as in-

83.

Synthesis of Arginine



Synthesis of Diaminopimelic Acid and Lysine

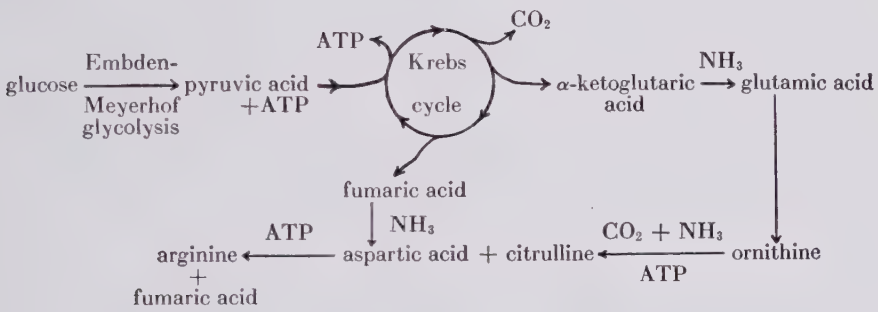


dicated above. The second amino group comes from glutamic acid via transamination.

Histidine. The imidazole ring of histidine is formed in part from adenosine monophosphate and in part from ribose-5-phosphate (see **85**). The imidazole glycerol phosphate so formed is then converted to histidine as shown in **86** on the following page.

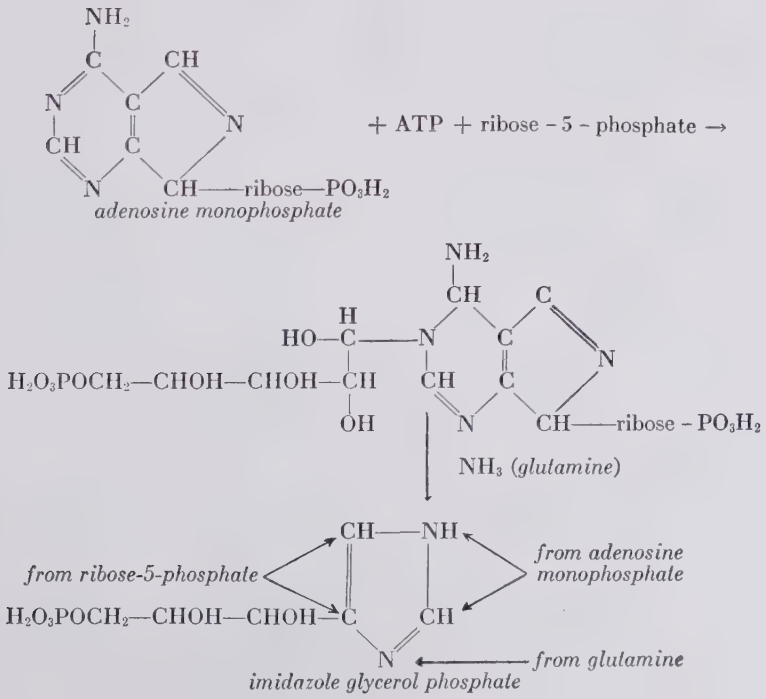
Aromatic amino acids. The amino acids which contain aromatic rings, phenylalanine, tyrosine, and tryptophan, are synthesized in *E. coli* from a common nonaromatic precursor derived from glucose. The details of these syntheses have been worked out only in *E. coli*, chiefly

84.



85.

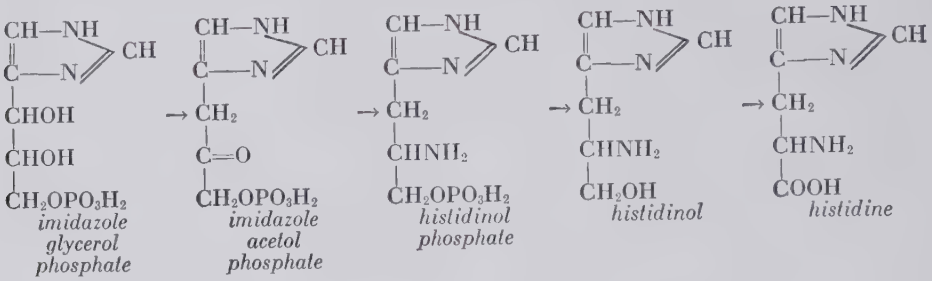
Synthesis of Imidazole Glycerol Phosphate



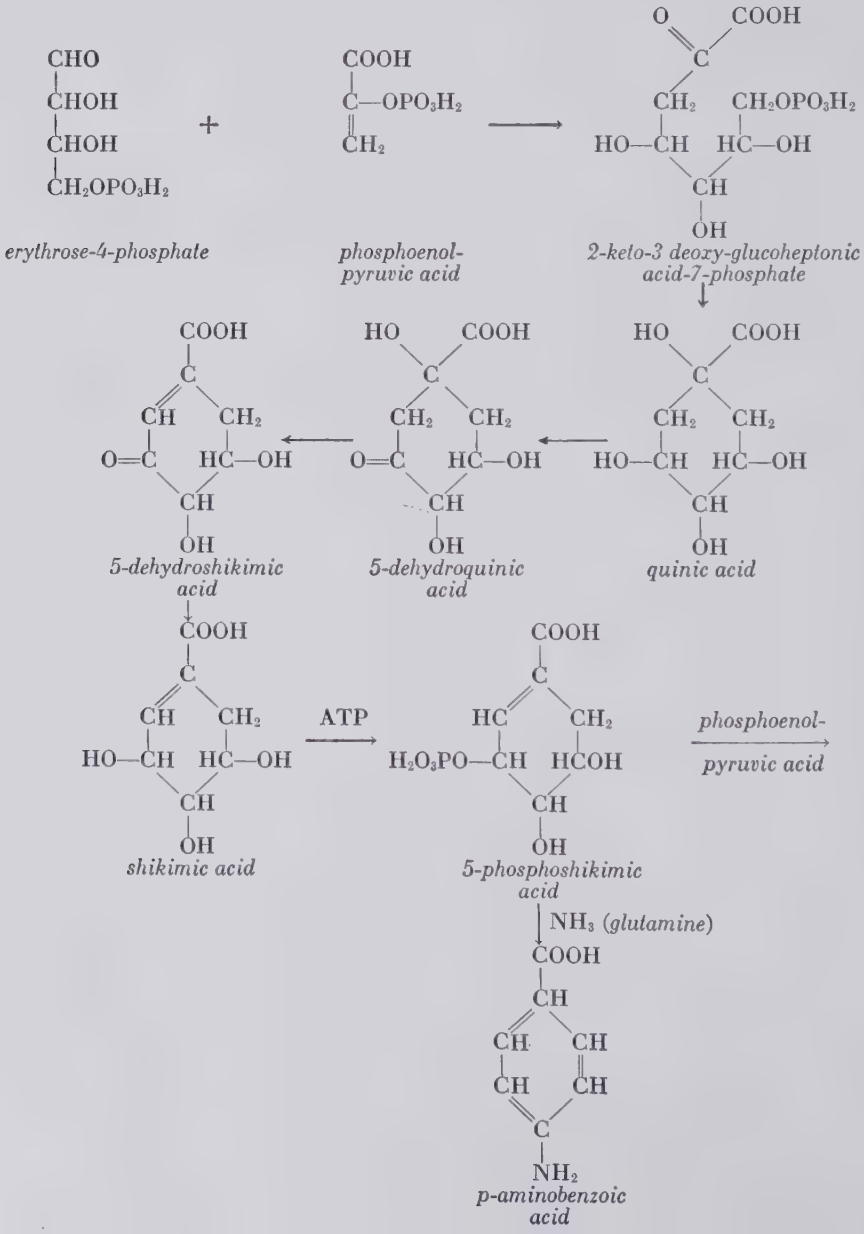
by use of biochemical mutants blocked at some stage in the synthetic pathway, but the general features of the mechanism seem to be of widespread distribution.

The first cyclic compound to be posi-

86. Synthesis of Histidine



87. Synthesis of the Aromatic Amino Acids



tively recognized is 5-dehydroquinic acid (see 87). Quinic acid is perhaps its immediate precursor. The cyclic compound is formed by the condensation of erythrose-4-phosphate and phosphoenolpyruvic acid. 2-Keto-3-deoxy-glucoheptonic acid-7-phosphate is probably an intermediate. 5-Dehydroquinic acid is converted by way of 5-dehydroshikimic acid to shikimic acid, which in turn produces 5-phosphoshikimic acid. At this stage, the synthetic pathways diverge along two independent routes. One leads to the three aromatic amino acids, and a second produces *p*-aminobenzoic acid.

General Consideration of Amino Acid Synthesis. Diagram 88 on the following page summarizes the biosynthetic pathways just discussed and also indicates the origin of several amino acids not specifically considered elsewhere. It is immediately obvious that there are three principal pathways from the carbon skeleton of glucose (and other carbohydrates) to the carbon skeletons of the amino acids:

(1) By way of the tricarboxylic acid cycle to α-ketoglutaric acid and oxalacetic or fumaric acids and thus to glutamic and aspartic acids.

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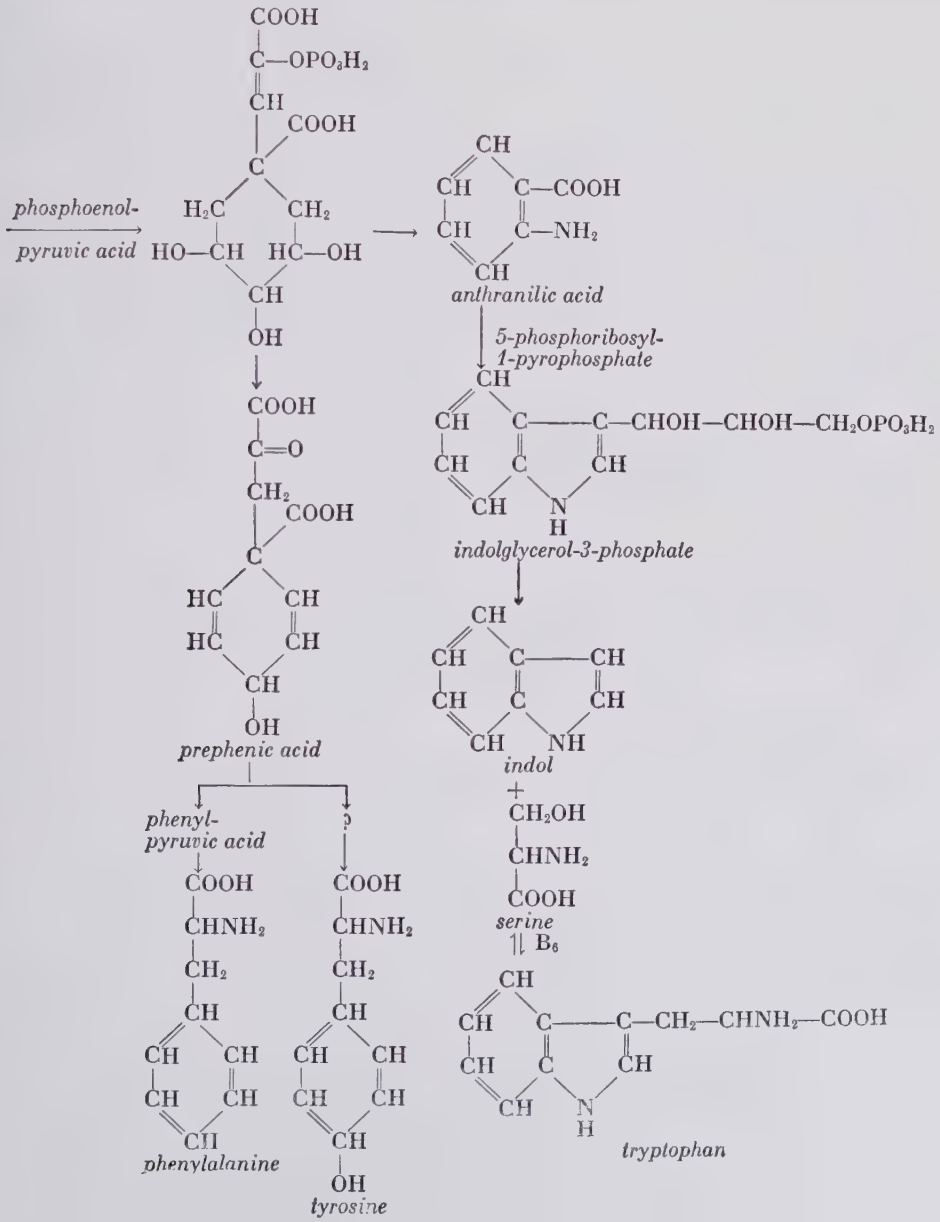
87. **Synthesis of the Aromatic Amino Acids (cont.)**



(2) By way of the pentose shunt to ribose phosphate and shikimic acid.
(3) By way of Embden-Meyerhof glycolysis to 3-phosphoglyceric acid and pyruvic acid.

The α-amino group is most commonly derived by:

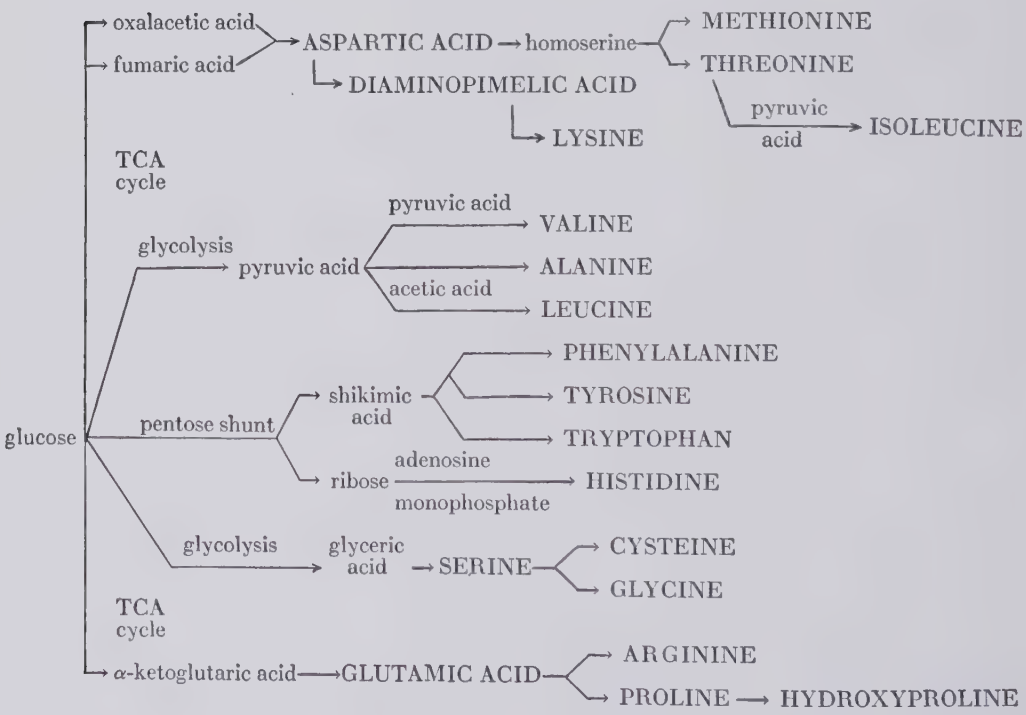
(1) Transformation of the carbon skeleton of glutamic or aspartic acids without loss of the α-amino groups.
(2) Transamination of the α-keto analogue of the amino acid as the last step in its synthesis.

With a few important exceptions, the modes of amino acid biosynthesis appear to be the same in all bacteria.

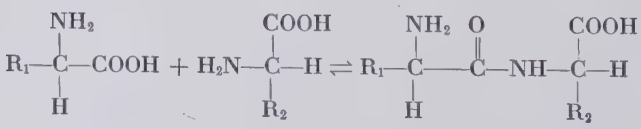
Synthesis of Peptides. The basic chemical reaction in the synthesis of both peptides and proteins is the synthesis of the peptide bond (see 89 below). The equilibrium of this reaction is far to the side of cleavage of the peptide bond, and therefore its synthesis requires an external source of energy. For all the peptides whose synthesis has been studied in detail, this energy is supplied by ATP which reacts with the

88.

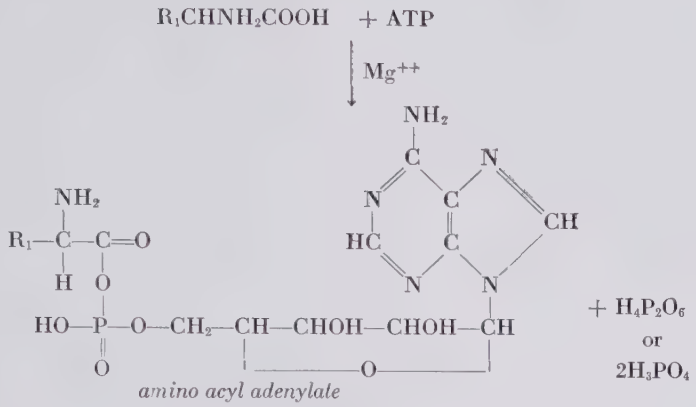
Biosynthetic Origin of Amino Acids



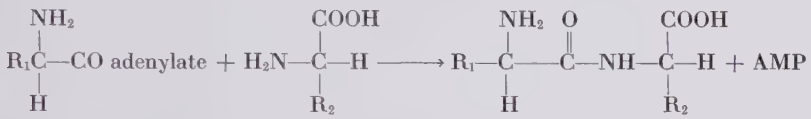
89.



90.



91.



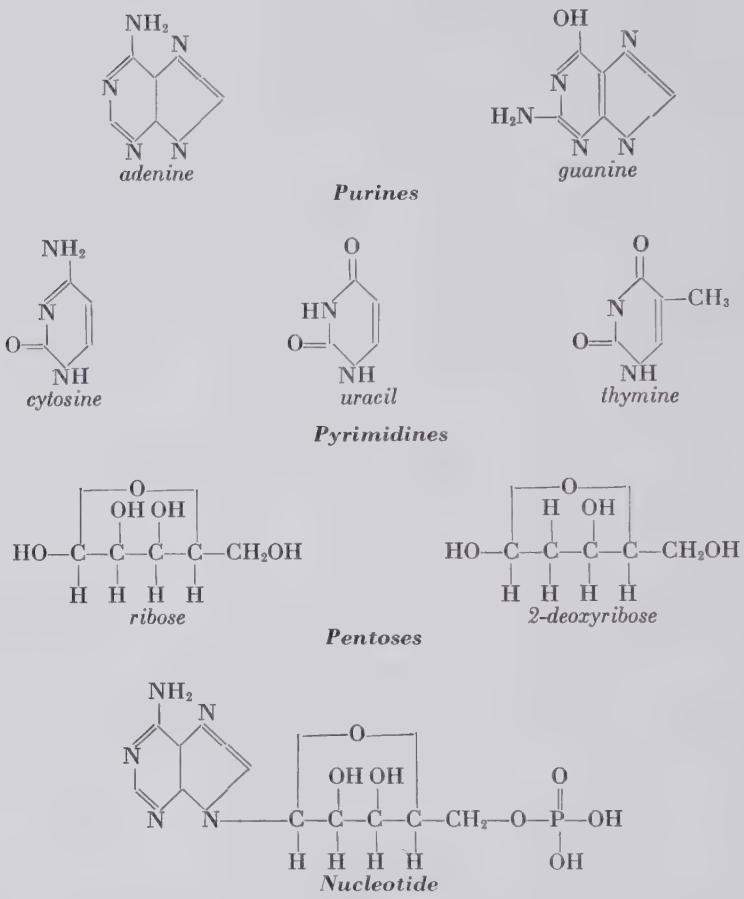
carboxyl group of one amino acid (or peptide) to form an amino acyl adenylate (see **90**). This carboxyl activation of amino acids by ATP has more than a superficial resemblance to the carboxyl activation of fatty acids by coenzyme A. This activated intermediate then reacts with another amino acid (or another suitable acceptor) to form a new peptide bond (see **91**).

Synthesis of Proteins. The synthesis of proteins and nucleic acids will be considered in a separate section near the end of this chapter.

METABOLISM OF NUCLEIC ACIDS AND RELATED SUBSTANCES

The main representatives of the second great group of naturally occurring nitrogenous substances are the nucleotides and the nucleic acids, which are high molecular weight substances made up of many nucleotide units.[59] A *nucleotide* is composed of a *purine* or *pyrimidine* base, a pentose sugar which is either *ribose* or *deoxyribose,* and a molecule of phosphoric acid (see structural formulas in **92** shown on page 182). Nucleotides are important as metabolic intermediates and as portions of coenzymes such as ATP and ADP, DPN and TPN, and CoA. *Nucleic acids* are of two main types. The *deoxyribonucleic acids* (DNA) contain deoxyribose, occur only in the cell nucleus, and function as the hereditary material. The ribose-containing *ribonucleic acids* (RNA) are found in both nucleus and cytoplasm and are closely associated with the synthesis of protein. Deoxyribonucleic acids contain adenine, guanine, cytosine and thymine, while ribonucleic acids have uracil in place of thymine.

92. Structural Components of Nucleic Acids



As with the proteins, there are many different nucleic acids, varying from cell to cell and within the individual cell, each with a unique structure and a unique biological function. Bacteria and other microorganisms have large amounts of nucleic acid as compared to higher forms. The RNA content is highly variable and dependent upon the physiological state of the bacterial cell.[7] Nucleic acids may occur in nature in combination with proteins as *nucleoproteins*. Nucleic acids and the various substances related to them contribute only a small portion of the total nitrogen of the usual bacteriological medium and are not generally a principal source of energy, carbon, or nitrogen. However, certain bacteria vigorously attack purines and pyrimidines and can use them as carbon and nitrogen sources. Also, some fastidious bacteria cannot synthesize all their purines and pyrimidines and must have them supplied in the growth medium.

BREAKDOWN OF NUCLEIC ACIDS[53]

Nucleoproteins are easily dissociated into nucleic acid and protein, and no specific enzymes for this cleavage have been described. All cells, bacteria included, have enzymes for degrading nucleic acids to smaller fragments. In both DNA and RNA, the nucleotides are linked in polynucleotide chains by

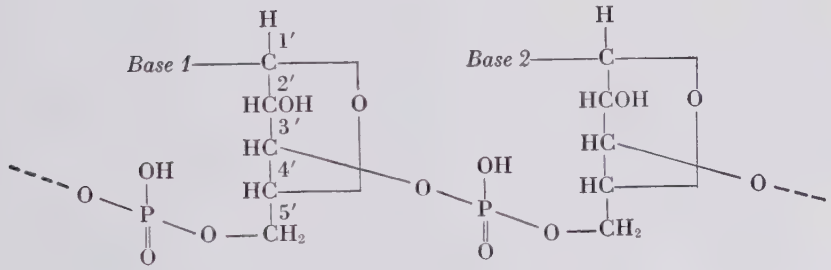
3′-5′-phosphate bridges (shown in **93** below, together with a convenient shorthand notation for representing a polynucleotide chain [**94**]). *Nucleodepolymerases* attack intact nucleic acids. *Deoxyribonucleases* (DNase) hydrolyze phosphate ester linkages in DNA. DNase I of pancreatic origin and streptococcal DNase (streptodornase) split 3′-bonds to yield 5′-mononucleotides and oligonucleotides with 5′-monophosphate ester end groups. On the other hand, DNase II, widely distributed in animal tissues and microorganisms, attacks 5′-bonds to yield 3′-fragments. All these enzymes fail to attack large portions of the DNA molecule, leaving a nonreactive core or limit polynucleotide.

Ribonucleases (RNase) appear to be of a single type. They hydrolyze 5′-phosphate esters of pyrimidine nucleotides to yield mononucleotides representing almost all the pyrimidines present, di- and trinucleotides, and a limit polynucleotide.

Phosphodiesterases hydrolyze -O-P-O- bonds in RNA and DNA. RNA is attacked directly, DNA only after exposure to DNase. *Venom diesterase*, found in the venoms of the rattlesnake and cobra families, splits 3′-bonds. It hydrolyzes RNA to 5′-mononucleotides and also reduces the oligonucleotides produced by DNase I to 5′-mononucleotides. Spleen diesterase splits 5′-bonds. Therefore, it produces 3′-mononucleotides from RNA and hydrolyzes to 3′-mononucleotides the oligonucleotides resulting from the action of DNase II on DNA.

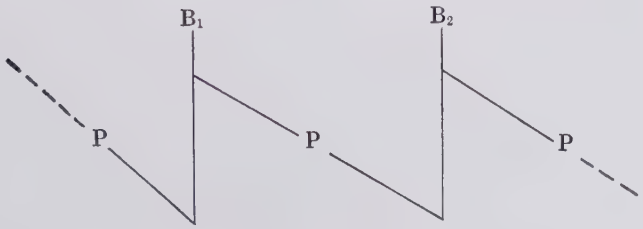
Phosphomonoesterases hydrolyze nucleotides to nucleosides and inorganic phosphate.

93.



3′—5′—Linkage of polynucleotides

94.



Shorthand notation for the structure above

The action of all these enzymes on the polynucleotide chain is summarized in **95**.

Nucleosides are cleaved to the free purine or pyrimidine base by rupture of the glycosidic bond between the nitrogen atom of the base and the sugar (N-glycosidic bond).

$$\text{nucleoside} + H_2O \xrightarrow{\text{nucleosidase}} \text{base} + \text{pentose}$$

Like the glycosidic bond between two sugars, this N-glycosidic bond may also be split by phosphorylases or transglycosidases. These reactions will be discussed in connection with the synthesis of nucleosides and nucleotides.

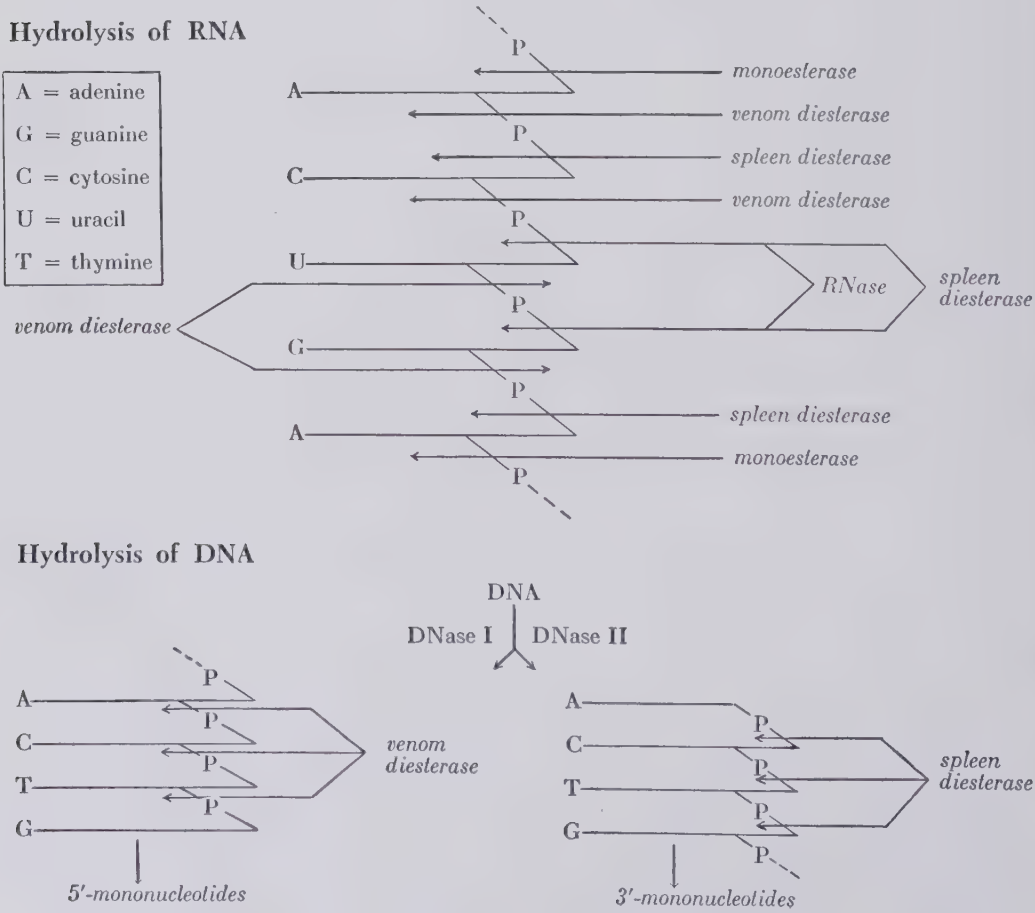
Some clostridia, micrococci, and other soil organisms oxidize free purines and pyrimidines and may even be able to use them as sole sources of carbon. Purines are broken down to glycine, acetic acid, formic acid, and CO_2, while pyrimidines yield β-alanine, lactic acid, acetic acid, and CO_2.[4]

The free *pyrimidines* are attacked by certain soil microorganisms and oxidized completely to CO_2 and H_2O. Barbituric acid, urea, and malonic acid may be intermediates in the oxidation.

The further metabolism of the *pentoses* released upon breakdown of nucleosides has been previously considered (see Carbohydrate Metabolism).

95.

Hydrolysis of Polynucleotides



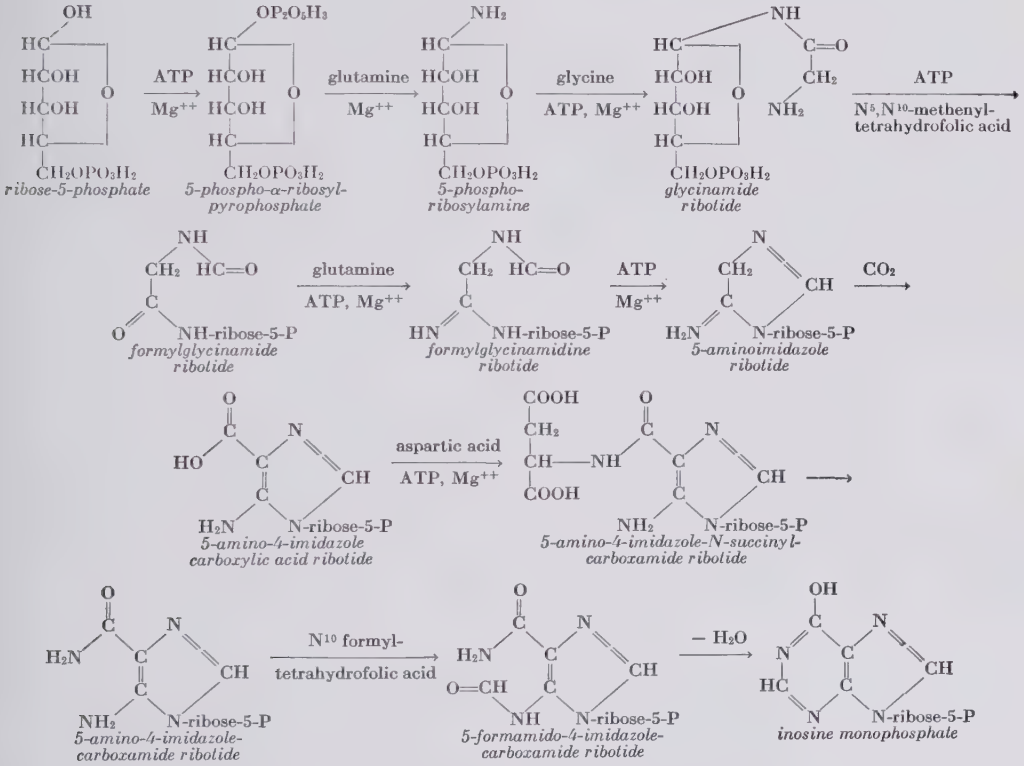
PRIMARY SYNTHESIS OF PURINES AND PYRIMIDINES

Although purines and pyrimidines are structurally related (see **92**), they are synthesized by completely independent pathways.

Synthesis of Purines.[10, 38, 54] In **96** are shown the reactions in the biosynthesis of purines. This biosynthetic pathway leads to inosine monophosphate, the ribose nucleotide of hypoxanthine. The free purine hypoxanthine is not an intermediate in the synthesis of its nucleotide because the purine ring is built onto ribose-5-phosphate, not vice versa. Following the initial condensation of glycine with 5-phosphoribosylamine, the remainder of the purine ring is formed by the stepwise addition of single atoms of carbon and nitrogen (see **96** and **97**).

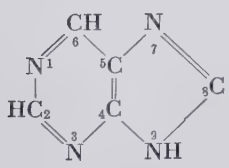
96.

Synthesis of Purines



97.

Origin of the Carbon and Nitrogen Atoms of the Purine Ring



	Carbon	*Nitrogen*
	C_4 and C_5—glycine	N_3 and N_9—glutamine
	C_2 and C_8—1-carbon donors via tetrahydrofolic acid	N_1—aspartic acid
	C_6—CO_2	N_7—glycine

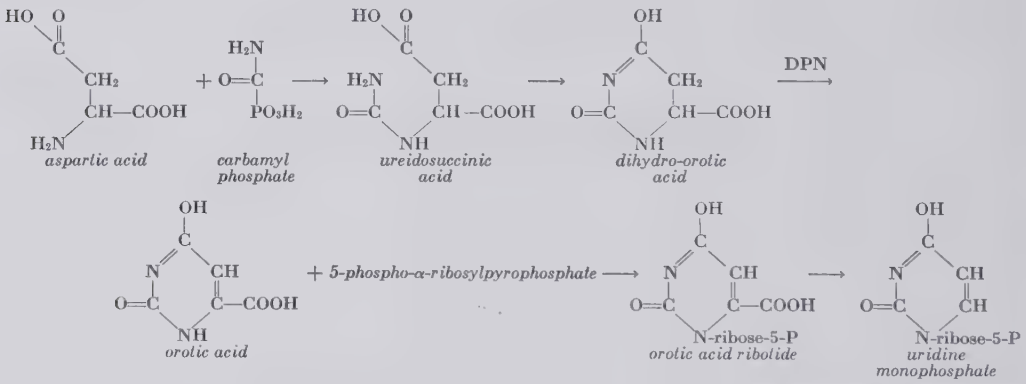
Synthesis of Pyrimidines.[13, 52, 54] Diagram **98** shows how pyrimidines are made. The synthetic mechanisms contrast sharply with those responsible for the production of purines. Aspartic acid and carbamyl phosphate condense to form the skeleton of the pyrimidine ring in one step, the ring is closed, and then the ribose-5-phosphate residue is added by reaction with 5-phospho-α-ribosylpyrophosphate. In an alternate pathway, β-alanine and carbamyl phosphate may condense to form uracil.

FURTHER METABOLISM OF PURINES AND PYRIMIDINES[59]

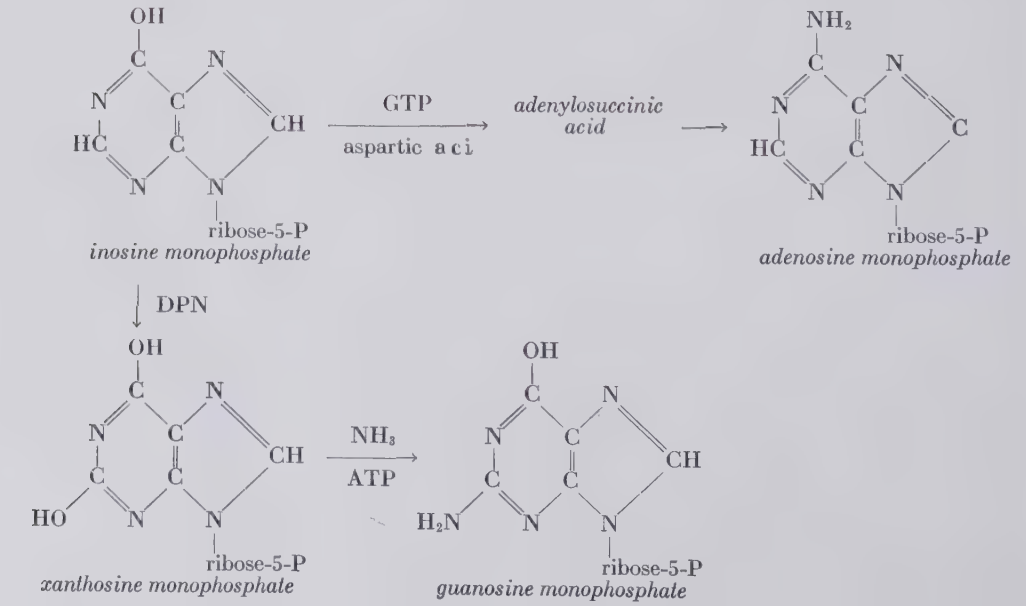
Bacteria do not independently synthesize each purine and pyrimidine and its corresponding ribonucleosides and deoxyribonucleosides and nucleotides. Instead they carry out extensive interconversions among these different types of purine and pyrimidine derivatives.

A number of different kinds of enzymes are responsible for these interconversions. In the adenine series, as a specific example, adenosine monophosphate is formed from inosine monophosphate by a reaction (see **99** below) in which aspartic acid is the nitrogen donor (cf. synthesis of arginine from citrulline). It may be split to adenosine and inorganic phosphate by a specific *nucleotidase*. Adenosine in turn may be cleaved to free adenine and ribose by a *nucleosidase*. Adenosine monophosphate and

98. Synthesis of Pyrimidines



99.



adenosine diphosphate are formed by the interaction of ATP and adenosine.

$$\text{adenosine} + \text{ATP} \xrightarrow[\text{kinase}]{\text{nucleoside}} \text{AMP} + \text{ADP}$$

Adenosine triphosphate is formed by oxidative phosphorylation as already described. The further metabolism of uridine monophosphate is shown in **100**.

Transformations involving alterations in the ring substituents of the purine and pyrimidine bases occur in several ways. The amino group of adenine is deaminated to the hydroxyl group of hypoxanthine in free adenine, adenosine, or adenosine monophosphate. Guanine may be similarly deaminated to xanthine and hypoxanthine reversibly oxidized to xanthine. In the pyrimidines, cytosine is deaminated to uracil and thymidine is demethylated to deoxyuridine.

A group of enzymes called *nucleoside phosphorylases* brings about the formation of new nucleosides by interchange of the nitrogen bases (see **101**).

Bacteria contain nucleoside phosphorylases active with both purine and pyrimidine bases and both ribose and deoxyribose, so these enzymes are capable of forming a wide variety of nucleosides.

Another group of enzymes, the *transglycosidases*, catalyzes the interconversions of deoxyribotides (see **102**).

The precise mechanism responsible for the synthesis of deoxyribonucleotides is still unknown. Deoxyribose may be directly synthesized by way of the following reaction:

$$\text{3-phosphoglyceraldehyde} + \text{acetaldehyde} \rightarrow \text{deoxyribose-5-phosphate}$$

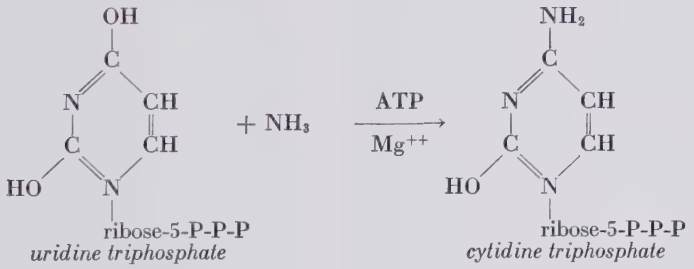
However, the principal pathway of deoxyribonucleotide synthesis appears to be the reduction of ribonucleotides to deoxyribonucleotides. In isotopic labeling experiments, the labeling pattern of ribose and deoxyribose is the same, and ribonucleotides may be converted to

---

100.

(*a*) $\text{UMP} + \text{ATP} \rightarrow \text{UDP} + \text{ATP}$
$2\ \text{UDP} \rightarrow \text{UTP} + \text{UMP}$

(*b*)



uridine triphosphate *cytidine triphosphate*

101.

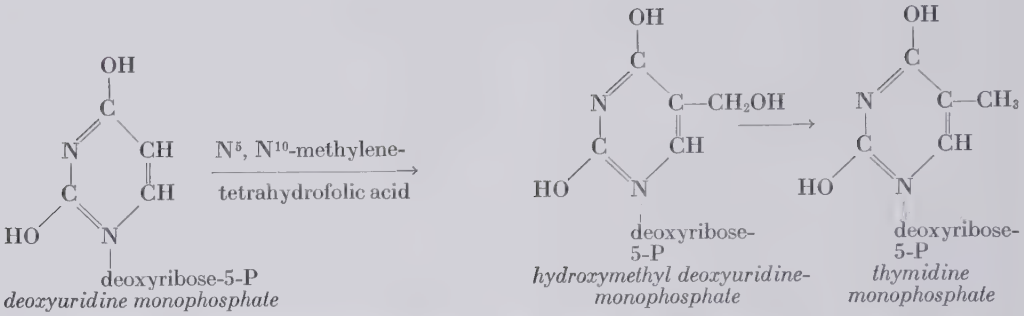
$$\text{Base A-pentose (nucleoside A)} + \text{H}_3\text{PO}_4 \underset{\text{phosphorylase A}}{\overset{\text{nucleoside}}{\rightleftharpoons}} \text{Base A} + \text{pentose-1-P}$$

$$\text{Base B} + \text{pentose-1-P} \underset{\text{phosphorylase B}}{\overset{\text{nucleoside}}{\rightleftharpoons}} \text{Base B-pentose (nucleoside B)} + \text{H}_3\text{PO}_4$$

102.

$$\text{Base A-deoxyribose-5-P (nucleotide A)} + \text{Base B} \rightleftharpoons \text{Base A} + \text{Base B-deoxyribose-5-P (nucleotide B)}$$

103.



the corresponding deoxyribonucleotides without breaking the sugar-base bond. Vitamin B_{12} is involved in this reduction.

Thymine nucleotides, which exist only as the deoxyribonucleotides, are formed by methylation of uridine monophosphate by a folic acid coenzyme (see **103**).

SYNTHESIS OF NUCLEIC ACIDS AND PROTEINS

All living cells contain the same low molecular weight building blocks produced by the same biosynthetic pathways. While some small molecules and some pathways occur uniquely in restricted groups of cells, variation in small-molecule content is not an important determinant of the individuality of cells. Individuality, which is just as important a biological principle as similarity, is primarily a function of the macromolecules of the cells—DNA, RNA, and protein. They are polymers of common low molecular weight units, the nucleotides and the amino acids, but these units are assembled in specific linear sequences, and it is in these unique sequential arrangements of a limited number of similar units that biological specificity resides.

The ultimate repositories of biological specificity (*i.e.*, genetic information) are the nucleotide sequences of molecules of DNA, the self-replicating genetic material. In addition to making more molecules like itself, the DNA causes the formation of RNA molecules whose nucleotide sequences carry some of the information possessed by the DNA. These RNA molecules in turn bring about the synthesis of the protein enzymes required for the energetic and biosynthetic functions of the cell. In recent years, there has been an explosive increase in our understanding of the molecular basis of these biosyntheses and the transfer of genetic information accompanying them, and it is now possible to give an explanation which, although still incomplete, is consistent and intellectually satisfying.

Structure and Self-duplication of DNA.[16, 60] According to the model of Watson and Crick, each DNA molecule consists of two polynucleotide chains coiled about a common axis. This double helix is held together by hydrogen bonds between pairs of bases (**104** on the opposite page). Because of structural limitations, hydrogen bonds are formed only between adenine and thymine and between guanine and cytosine (**105** on the opposite page).

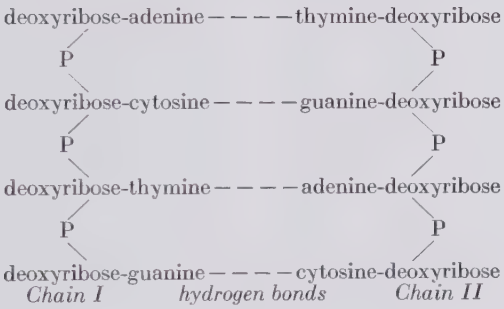
In a single chain of DNA, any sequence of nucleotides is possible, and hence a very large number of different sequences, each carrying unique genetic information, is also possible. However, if

the order of bases in one chain is specified, the exact sequence in the other chain is simultaneously determined because of the restrictions on base pairing. Thus, each chain in a molecule of DNA is the complement of the other, and each molecule contains two chains but only a single code.

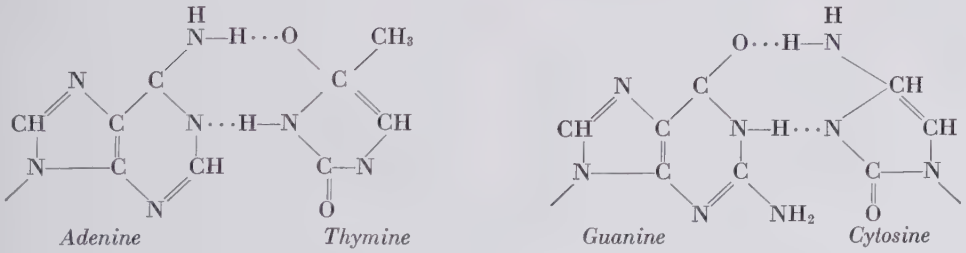
This complementary structure of the double helix is the key to the mechanism of duplication of DNA. This duplication may be visualized most simply by assuming that the two chains unwind and separate. Each chain then acts as a template for the formation of a new chain with complementary nucleotide sequence. The net result is that two new chains are produced, complementary to each other and each identical with one of the original chains as well.

Synthesis of DNA.[37] *Escherichia coli* contains an enzyme which catalyzes the formation of polynucleotides with properties very similar to, if not identical with, DNA isolated from natural sources. The polymerization may be visualized as in **106.** All four deoxyribonucleoside triphosphates are required. Pyrophosphate is released, and the energy of the nucleoside triphosphates is used to form the polynucleotide chain. Mg^{++} is necessary for the reaction, and a small amount of polymerized DNA must be present to act as a primer, presumably in

104.



105.



Pairing of Bases in DNA

106.

$$n\begin{bmatrix} dTPPP \\ dGPPP \\ dAPPP \\ dCPPP \end{bmatrix} \xrightarrow[Mg^{++}]{DNA} \begin{bmatrix} dTP \\ dGP \\ dAP \\ dCP \end{bmatrix}_n + 4(n)PP$$

DNA

the manner just postulated for DNA replication. The amount of new DNA synthesized is many times that added as primer.

The newly synthesized DNA has all the physical properties of natural DNA and is hydrolyzed by pancreatic DNase. The ratios of adenine to thymine and of guanine to cytosine are those required by the Watson-Crick model, and the base ratios of the new DNA molecules are very close to those of the primer.

It has not yet been possible to synthesize biologically active DNA *in vitro*, probably because DNA fragments of relatively small size are as effective in priming the reactions as intact DNA molecules.

Synthesis of RNA. Bacteria, plants, and higher animals possess an enzyme which catalyzes the synthesis of RNA from all four ribonucleotide triphosphates, Mn^{++}, and a DNA primer (**107**).[58, 61]

The resemblance to the DNA-synthesizing system just described is striking. Here DNA also acts as a template for polynucleotide assembly. However, since the building materials at hand are ribonucleotides, RNA, not DNA, is produced. The template role of the primer DNA is clearly shown by observations that the composition of the newly synthesized RNA closely approximates that of the DNA primer, even when DNA molecules of widely varying base ratios are employed.

Although, as in the DNA system, biologically active RNA has not yet been made *in vitro*, there is good evidence that RNA is synthesized in the nucleus of the cell with DNA for a template, and that this "messenger RNA" then goes to the sites of protein synthesis in the cytoplasm where it in turn acts as a template for the assembly of the polypeptide chains of proteins.[26]

Ribopolynucleotides are also synthesized by the widely distributed polynucleotide phosphorylase.[24]

$$\mathrm{n\,[XPP] \overset{Mg}{\rightleftharpoons} [XP]_n + nP}$$

The enzyme polymerizes single ribonucleotide diphosphates or mixtures of them. Thus, polymers containing 1, 2, 3, or 4 nitrogen bases may be synthesized. When the polynucleotide phosphorylase is purified, a primer becomes necessary. The requirement is for an oligonucleotide with a free 3′-hydroxyl group to which the new nucleotide units are added.

While polynucleotide phosphorylase probably does not function in the biosynthesis of RNA *in vivo*, it has furnished a means for the synthesis of simple ribopolynucleotides, which, as will be discussed shortly, have been invaluable in elucidating the role of RNA in protein synthesis.

Synthesis of Proteins.[8, 41, 43] Protein synthesis requires a source of energy for formation of the peptide bond and a mechanism for ordering the sequence of amino acids in the polypeptide chain. As in the synthesis of DNA and RNA, the energy is supplied by a nucleoside triphosphate and the template by a polynucleotide. However, since proteins are made up of amino acids, not nucleotides, both processes are more indirect and more complicated.

Amino acids are activated in the form of aminoacyl adenylates by specific ac-

107.

$$\mathrm{n\begin{bmatrix}UPPP\\GPPP\\APPP\\CPPP\end{bmatrix} \xrightarrow[Mn^{++}]{DNA} \begin{bmatrix}UP\\GP\\AP\\CP\end{bmatrix}_n + 4(n)PP}$$

RNA

tivating enzymes associated with the cytoplasmic membrane and soluble fraction of the bacterial cell (see "Peptide Synthesis"):

$$AA + ATP \rightarrow AA\text{-}AMP + PP$$

The next step is the transfer of the activated amino acid to a special kind of RNA, *transfer* or *soluble* RNA.

$$AA\text{-}AMP + T\text{-}RNA \rightleftharpoons T\text{-}RNA\text{-}AA + AMP$$

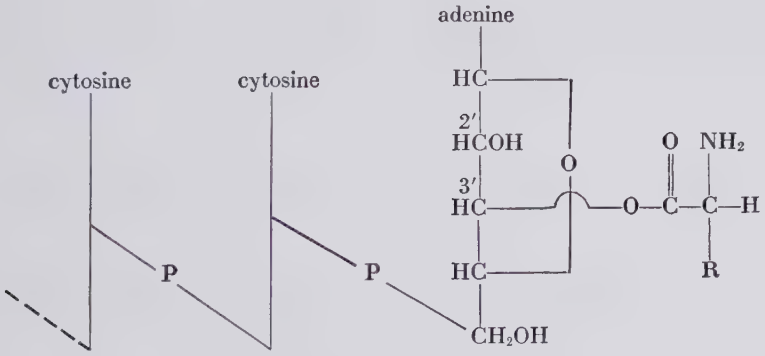
There appears to be a specific transfer RNA for each amino acid, which is apparently the same in all cells. All amino acids are linked to their transfer RNA's through esterification to the 2′- or 3′-hydroxyl groups of a terminal adenosine moiety. These esters have sufficient energy to allow the amino acids to enter into peptide bond formation.

The last three nucleotides of transfer RNA are always as shown below, those of cytosine, cytosine, and adenine (**108**). The remainder of the polynucleotide chain must have a sequence specific for a particular amino acid.

While the actual synthesis of protein in the mammalian cell occurs mainly in the ribonucleoprotein particles of the cytoplasm, the ribosomes, bacterial protein synthesis proceeds actively in both the cytoplasmic membrane and in the ribosomes. Messenger RNA is the template on which the amino acids are assembled to be formed into polynucleotide chains. It cannot function directly, however, since a polypeptide cannot be formed on a polynucleotide. It appears that the specific portion of the transfer RNA acts as an "adapter" and solves this difficulty. That is, the specific section of the transfer RNA molecule is complementary to a portion of the polynucleotide chain of the messenger RNA and hydrogen bonds to it, thus locating its activated amino acid in a definite relationship to other amino acids similarly bound. Once alignment of the required sequence of amino acids is achieved, peptide bond formation occurs and the newly synthesized protein is released (**109**).

This process requires a number of enzymes and cofactors whose function is not clearly understood, among these be-

108



Linkage of Amino Acids to Transfer RNA

109.

$$n\ T\text{-}RNA\text{-}AA \xrightarrow[M\text{-}RNA]{GTP} [AA]_n + T\text{-}RNA$$

(protein)

ing the so-called supernatant factor, GTP, and phosphoenolpyruvic acid and its kinase.

The complicated interrelationships in the biosynthesis of DNA, RNA and protein are shown in a greatly simplified and abbreviated diagram below (**110**). Note that the ultimate source of genetic information is the sequence of bases in DNA and that the ultimate source of biosynthetic energy is the high energy bonds of ATP.

Nature of the Genetic Code for Proteins.[14, 42, 44] A large amount of indirect evidence suggests that the genetic information required for the insertion of a specific amino acid into a specific location in a polypeptide chain is contained in three successive nucleotide residues of DNA and messenger RNA: that is, the genetic code for each amino acid consists of a nucleotide triplet. This concept has received direct confirmation by the observation that synthetic ribopolynucleotides produced *in vitro* by polynucleotide phosphorylase can substitute for messenger RNA and stimulate protein synthesis (as measured by uptake of labeled amino acids) in ribosomes of *Escherichia coli*. Stimulation of amino acid incorporation is highly specific, and from a large number of experiments with synthetic nucleotides containing one, two or three different bases, it has been possible to determine a triplet code for each amino acid as shown in the accompanying table.

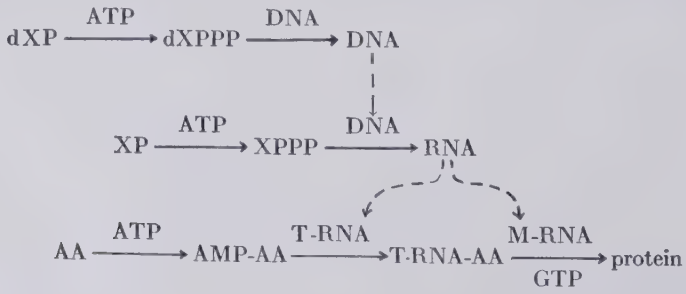
The Triplet Code for Amino Acids

AMINO ACID	NUCLEOTIDE TRIPLET (SEQUENCE UNKNOWN)
Alanine	UCG
Arginine	UCG
Aspartic acid	UAG
Cystine	UUG
Glutamic acid	UAG
Glycine	UGG
Histidine	UAC
Isoleucine	UUA
Leucine	UUC
Lysine	UAA
Methionine	UAG
Phenylalanine	UUU
Proline	UCC
Serine	UUC
Threonine	UAC
Tryptophan	UGG
Tyrosine	UUA
Valine	UUG

When two amino acids have the same code designation, this may be because their codes contain the same three nucleotides in different sequences or it may mean that the code is degenerate; *i.e.*, more than one amino acid may be specified by the same triplet. The universal appearance of uracil in the codes is of unknown significance. Other evidence suggests that the triplet code is nonoverlapping, that it is read in one direction from a single starting point, and there are no "punctuation marks" between successive triplets.

110.

Interrelationships in the Synthesis of Nucleic Acids and Proteins



NUTRITION OF BACTERIA

In order to synthesize the many components of their protoplasm and thus to grow and multiply, bacteria must be supplied with a medium containing the proper chemical constituents for supporting the necessary synthetic reactions. The chemical substances which must be furnished bacteria in order that they may grow and multiply are known as their *growth requirements* or *nutritional requirements*. These nutritional factors are usually classified as:

(1) Compounds required as energy sources.
(2) Compounds required as building stones for the synthesis of new protoplasm.
    (a) Compounds required as carbon sources.
    (b) Compounds required as nitrogen sources.
    (c) Organic compounds required in their intact form as growth factors.
    (d) Inorganic ions required for metabolism and growth.

Given these necessary factors, bacteria will grow in the proper physico-chemical environment (favorable temperature, pH, O_2 tension, etc.). Different kinds of bacteria have widely varying growth requirements, and even the same bacterium may have variable nutritional needs, depending upon the conditions of growth and the presence or absence of other substances in the medium. Specific examples of such phenomena will be considered shortly.

Many aspects of bacterial nutrition have already been treated in terms of the metabolic functioning of the various nutritional factors. In particular, the sources of energy, carbon, and nitrogen have been repeatedly discussed and will not be considered further here. Much has also been said about the metabolic function of growth factors, so the chief object of this section will be to collect and summarize the basic information about the role of growth factors in bacterial nutrition.

Growth Factors. A *growth factor* may be defined as an organic compound which a bacterium needs for growth yet cannot synthesize. Since all cells have essentially the same chemical make-up, it follows that bacterium A, which does not need a growth factor required by bacterium B, lacks this requirement because it can synthesize the factor in question. Thus, *L. casei* requires folic acid, while *E. coli* does not because it makes its own folic acid. This concept has been proved many times. Therefore, nutritional requirement may be equated to synthetic disability.

It is generally believed that the variations in nutritional requirement observed among presently existing groups of bacteria have arisen by loss of synthetic ability through genetic modifications. This idea receives direct experimental support from work in bacterial genetics (Chap. 7) which has demonstrated that mutations in bacteria cause the loss of ability to synthesize precisely those compounds—the B vitamins, amino acids, purines, and pyrimidines—which are growth factors for naturally occurring bacterial populations.

Growth factors are of two main types: (1) those required in minute amounts and which function catalytically as portions of enzyme systems: the B vitamins are the principal representatives of this type; (2) those required in substantially large quantities and which are incorporated directly or with only minor modifications into cell material: the amino acids, purines, and pyrimidines are the chief examples of this kind of growth factor.

An organism may exhibit an *absolute requirement* for a growth factor; *i.e.*, no growth at all occurs in its absence. On the other hand, a growth factor may be *stimulatory; i.e.*, limited growth without

the factor is sharply increased upon its addition. Examples of these types of requirements will be given presently.

The Vitamin B Group. The B-complex vitamins or the vitamin B group are low molecular weight, water-soluble organic compounds which are universal constituents of all living cells, in which they function as coenzymes or portions of coenzymes. It is customary to designate as the vitamin the simplest compound which, when supplied to the cell, allows it to synthesize the coenzyme. Thus, pantothenic acid is a B vitamin whose coenzyme form is Co A. Some B vitamins, such as nicotinic acid and thiamin, were discovered from studies of animal nutrition, while others, such as pantothenic acid and vitamin B_{12}, were first recognized as microbial growth factors. The complete identity of the vitamin B requirements of microorganisms and higher animals is now so well established that when a new bacterial growth factor is found, its role in animal metabolism is immediately searched for, and vice versa.

Since the B vitamins function catalytically, they promote bacterial growth in very low concentrations. The B vitamins are usually supplied to bacteria in concentrations of about 10^{-6} to 10^{-10} M or 0.0001 to 0.00000001 mg. per ml. of medium. Vitamin B_{12} and biotin are generally required in smallest amount, nicotinic acid and riboflavin in the largest.

The table above summarizes the metabolic function of the B vitamins.

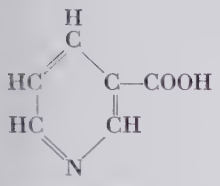
Thiamin.[1, 34] Thiamin is one of the earliest recognized and most frequently required bacterial growth factors. It is synthesized by condensation of the thiazole portion and the pyrimidine portion of the molecule (see **111** on the opposite page).

As expected from the way thiamin is synthesized, some bacteria require the thiazole alone, the pyrimidine alone, both thiazole and pyrimidine, or intact thiamin. The biosynthetic origin of the thiazole and pyrimidine intermediates is unknown. So far, no microorganism has been found with a requirement for the whole coenzyme, DPT.

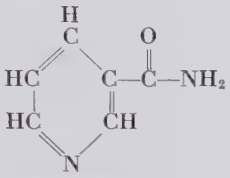
Metabolic Functions of the Vitamin B Group

B VITAMIN	TYPICAL ORGANISMS REQUIRING IT	COENZYME FORM	METABOLIC FUNCTIONS
thiamin	*Staph. aureus* *L. fermenti*	DPT	activation of keto acids and keto sugars; transfer of 2-carbon units
nicotinic acid	*L. arabinosus* *Pr. vulgaris*	DPN and TPN	hydrogen transfer
riboflavin	*L. casei* *Str. lactis*	riboflavin-5-P flavin-adenine-dinucleotide	hydrogen transfer
vitamin B_6			
pyridoxal	*L. casei*	pyridoxal-P	decarboxylation, deamination, transamination, and racemization of amino acids
pyridoxal or pyridoxamine	*Cl. welchii* *Str. fecalis*	pyridoxamine-P	
pantothenic acid	*Brucella abortus* *Pr. morganii*	Co A	acyl activation and transfer
p-aminobenzoic acid	*Cl. acetobutylicum* *Acetobacter suboxydans*	tetrahydrofolic acid	1-carbon-transfer
folic acid	*L. casei* *Cl. tetani*	tetrahydrofolic acid	
biotin	*Leuc. mesenteroides* *Cl. tetani* *L. arabinosus*	biotin-CO_2	CO_2 fixation, fatty acid synthesis
vitamin B_{12}	*L. leichmannii* *L. lactis*	(?)	1-carbon transfer synthesis of deoxyribosides

Nicotinic acid (niacin).[29, 30] Nicotinic acid and nicotinamide are the growth factors usually required by bacteria for the synthesis of the coenzymes DPN and TPN, which function in hydrogen transport (see "Respiration" for structures of DPN and TPN).



nicotinic acid



nicotinamide

However, many species of the genus Hemophilus cannot utilize nicotinic acid or its amide for the synthesis of DPN and TPN and must be supplied with the intact coenzymes or with nicotinamide riboside.

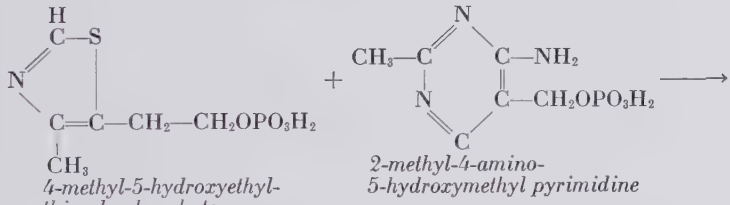
It is assumed that the bacteria which require nicotinic acid or the amide are unable to carry out the previously discussed conversion of tryptophan to nicotinic acid. The probable metabolic pathway from nicotinic acid to DPN and TPN is shown in **112** below.

It is of interest that nicotinamide reacts with ribose-1-P the same way as do purines and pyrimidines. It should also be noted that ATP has a dual function in pyridine nucleotide synthesis: (1) it acts as a phosphorylating agent (reactions 3 and 5) and (2) it acts as a donor of an adenosine monophosphate group to nicotinamide-ribose-5-P in the final synthesis of DPN (reaction 4).

Riboflavin.[6, 46] Riboflavin as a growth factor resembles nicotinic acid, not in structure, but in the functioning of its coenzyme in hydrogen transfer and in the mode of synthesis of the coenzyme

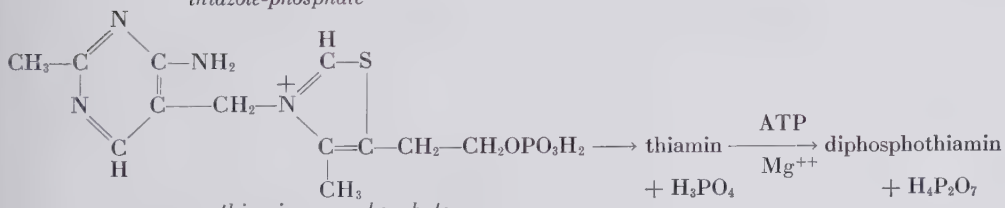
---

111.



4-methyl-5-hydroxyethyl-thiazole-phosphate

2-methyl-4-amino-5-hydroxymethyl pyrimidine



thiamin monophosphate

112.

(1) nicotinic acid + NH_3 → nicotinamide
(2) nicotinamide + ribose-1-P → nicotinamide riboside + H_3PO_4
(3) nicotinamide riboside + ATP → nicotinamide-5-P + ADP
(4) nicotinamide-5-P + ATP → DPN + P-P
(5) DPN + ATP → TPN + ADP

from the vitamin. Riboflavin has the structure shown in **113**.

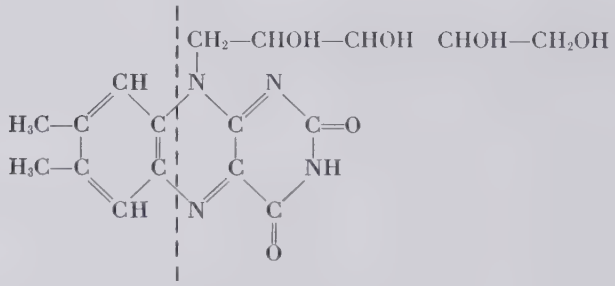
It is composed of a nitrogenous base, 6,7-dimethylisoalloxazine (flavin) and *ribitol,* the sugar alcohol corresponding to ribose, and is, therefore, comparable in structure to nicotinamide riboside. It is interesting that the free base itself is not a growth factor. Its coenzyme forms are riboflavin-5-phosphate and flavin-adenine dinucleotide (see "Respiration" for structures).

The biosynthesis of the isoalloxazine ring is closely related to that of the purine ring. Note that the portion of the isoalloxazine ring lying to the right of the dotted line in **113** is identical with that of the purine ring (minus carbon 8).

The biosynthesis of the isoalloxazine ring appears to be closely related to the formation of purines. The riboflavin coenzymes are synthesized in a manner quite similar to that of the nicotinic acid coenzymes (see **114**).

Vitamin B_6.[9] Vitamin B_6 is a collective term for several closely related factors (structural formulas, **115**). Of these, pyridoxal phosphate and pyridoxamine phosphate are the coenzyme forms (see transamination). For higher animals, yeasts and molds, pyridoxine, pyridoxal, and pyridoxamine are of equal activity as growth factors, but for most bacteria, either pyridoxal or pyridoxamine is required. Some, such as *L. casei,* respond only to pyridoxal, while a few

113.

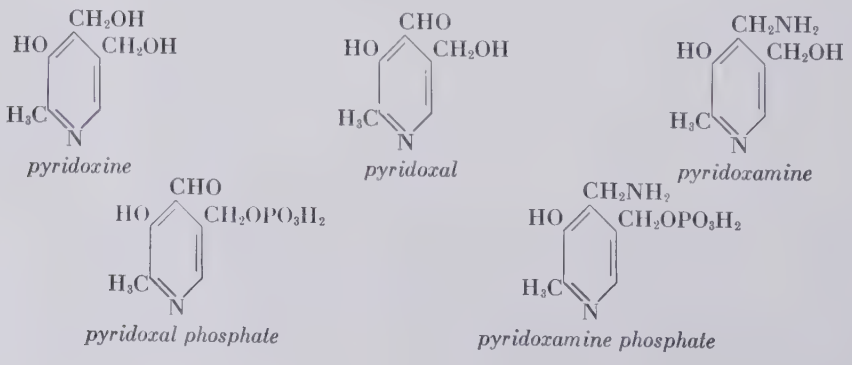


114.

riboflavin + ATP → riboflavin-5-P + ADP
riboflavin-5-P + ATP → flavin-adenine dinucleotide + P-P

115.

The Vitamin B_6 Family



strains of lactobacilli require either pyridoxal phosphate or pyridoxamine phosphate.

Vitamin B_6 is a good example of a growth factor which is highly stimulatory, but not absolutely required. Most yeasts and lactobacilli such as *L. arabinosus* will produce heavy growth in the absence of vitamin B_6, but in its presence the same amount of growth is achieved in a much shorter time.

Since it has been repeatedly demonstrated that pyridoxal phosphate is closely associated with almost every phase of amino acid metabolism, it is not surprising that the quantitative pyridoxine requirement depends upon the amino acid composition of the medium. This is particularly true for the lactobacilli which require many amino acids as growth factors. *L. arabinosus* can apparently synthesize a small amount of vitamin B_6, enough for growth on a medium rich in amino acids, such as a casein hydrolysate. However, omission of these amino acids forces the organism to make them itself, and it cannot synthesize enough vitamin B_6 to catalyze the reactions involved. Therefore, on a medium poor in amino acids, *L. arabinosus* must have additional vitamin B_6 for growth. The complete replacement of the vitamin B_6 requirement of *Str. fecalis* by D-alanine has already been pointed out (see "Racemization of Amino Acids").

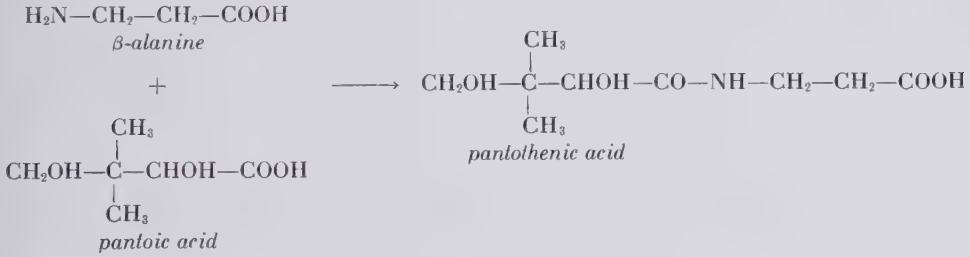
Pantothenic acid.[27] Pantothenic acid is a growth factor for a large number of bacteria. Its only known function is as a part of Co A, the coenzyme of acetyl transfer. It is synthesized by the union of β-alanine and pantoic acid, which are derived from aspartic acid and valine, respectively (see **116**).

As with thiamin, some organisms require only one portion of the vitamin (β-alanine or pantoic acid) for growth, but the lactobacilli and many other bacteria can utilize only intact pantothenic acid.

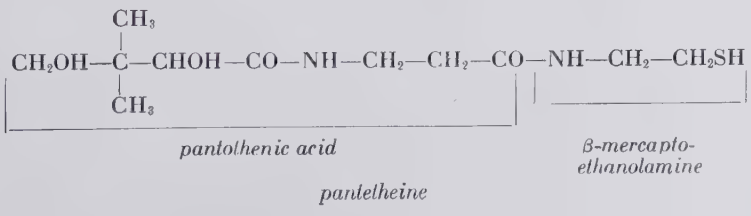
One lactobacillus, *L. bulgaricus*, requires a higher form of pantothenic acid, *pantetheine*, formed from pantothenic acid and cysteine (see formula **117**). It is very probable that pantetheine is an intermediate in the biosynthesis of Co A (see "Carbohydrate Metabolism" for structure of Co A) and that *L. bulgaricus* has lost the ability to unite the two halves of the pantetheine molecule.

Co A is not an absolute growth requirement for any microorganism, but it is a stimulatory factor for *Acetobacter suboxydans; i.e.*, the organism grows more rapidly in the presence of Co A than in that of pantothenic acid. Co A

116.



117.



is formed from pantetheine (see **A** below).

Note that ATP plays the same dual role as a donor of phosphate groups and of adenosine monophosphate groups as in the synthesis of the pyridine and flavin nucleotides.

***p*-Aminobenzoic acid and the folic acid group.**[28, 47, 49] The role of folic acid in 1-carbon transfer has already been discussed in connection with the metabolism of amino acids and of purines and pyrimidines. Each member of the folic acid group contains *p*-aminobenzoic acid and a pteridine nucleus, and they are metabolically interconverted by many bacteria. The biosynthetic pathway for *p*-aminobenzoic acid formation has already been discussed. The pteridine ring has a close structural resemblance to the purine and isoalloxazine rings, and all three are synthesized by related mechanisms.

p-Aminobenzoic acid is incorporated into folic acid, and the sulfonamides inhibit bacterial growth by preventing the incorporation.

Many bacteria, such as *E. coli*, do not require *p*-aminobenzoic acid or any of its higher forms, but its importance in their metabolism is easily demonstrated by its ability to overcome the growth inhibition produced by sulfonamides. Others, such as *L. arabinosus*, require preformed *p*-aminobenzoic acid but can synthesize the remainder of the folic acid coenzyme themselves. However, certain lactobacilli and clostridia also lack the ability to synthesize other portions of the folic acid molecule as well. Thus, *Str. fecalis* requires a growth factor of at least the complexity of pteroic acid, in which the bond between *p*-aminobenzoic acid and the pteridine nucleus has already been formed. *L. casei* lacks, in addition, the ability to join glutamic acid to pteroic acid and, therefore, requires pteroylglutamic acid as a growth factor. Finally, *Pediococcus cerevisiae* needs leucovorin which differs from pteroylglutamic acid in the possession of a formyl group and a reduced pteridine nucleus. There are also many other forms of folic acid. Some are active for one or more folic acid–requiring bacteria in their naturally occurring forms. Others, the folic acid conjugates, are active only after they are partially broken down by enzymes, conjugases.

The coenzyme forms of folic acid are tetrahydrofolic acid and derivatives of this compound with active 1-carbon groups. Their probable biosynthetic origin is shown in the accompanying diagram (**118** on the opposite page). Their function in the metabolism of purines, pyrimidines and amino acids has already been discussed. A generalized formulation of the role of folic acid coenzymes in 1-carbon transfer is shown in **B** below. Other evidence also suggests that folic acid may be active in the synthesis of the amino acids histidine, threonine, and leucine.

Just as the vitamin B_6 requirement is profoundly influenced by the amino acid composition of the medium, so may the *p*-aminobenzoic acid or folic acid requirement be reduced or abolished by addition of the purines, pyrimidines, and amino acids which are synthesized in folic acid–requiring reactions. These same substances will also counteract the growth-inhibiting action of sulfonamides on organisms which require neither *p*-aminobenzoic acid nor folic acid.

Biotin.[45] Biotin is a growth factor for many different types of microorganisms and for higher animals. It is likely that

A

pantetheine + ATP → 4′-phosphopantetheine + ADP
4′-phosphopantetheine + ATP → dephospho Co A + P-P
dephospho Co A + ATP → Co A + ADP

B

1. tetrahydrofolic acid + donor-C_1 ⇌ tetrahydrofolic acid-C_1 + donor
2. tetrahydrofolic acid-C_1 + acceptor ⇌ tetrahydrofolic acid + acceptor-C_1

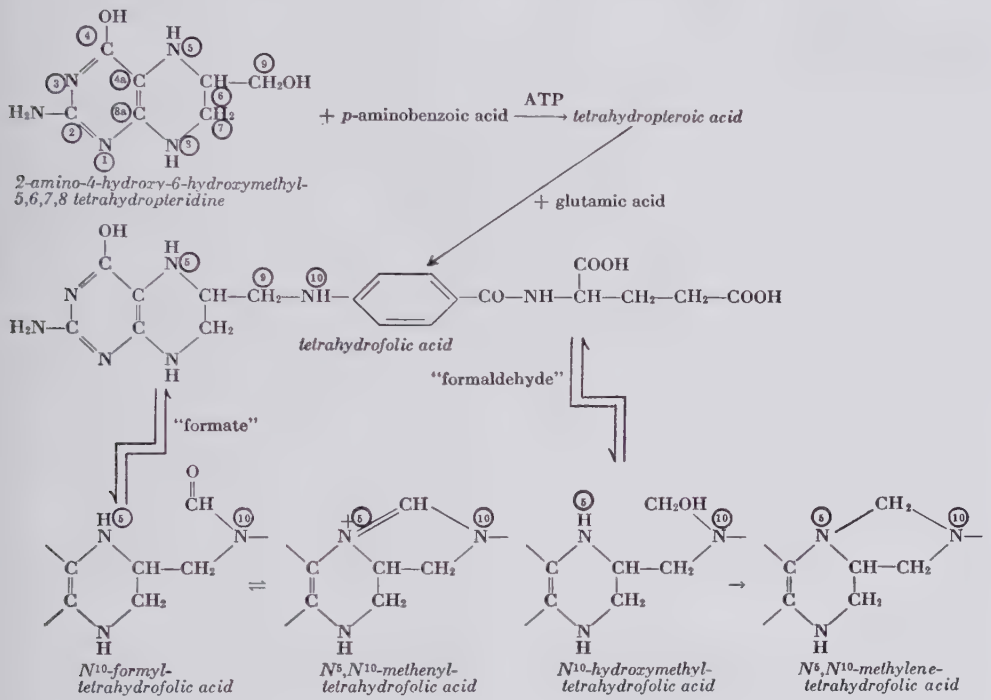
the pathway of biotin biosynthesis is as shown in **119**.

This scheme is based on the observations that in some bacteria, such as *Corynebacterium diphtheriae*, pimelic acid will replace the biotin requirement, and that in others, such as *Leuc. mesenteroides*, desthiobiotin is a precursor of biotin.

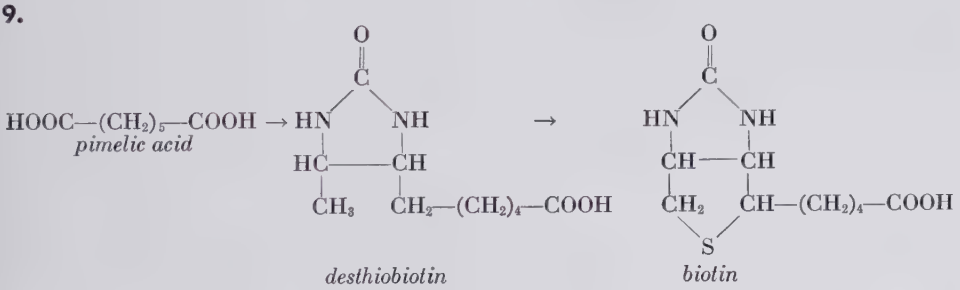
Naturally occurring biotin often exists in bound forms. One of these, *biocytin*, is a lysine derivative. (See **120**.) Bio-

118.

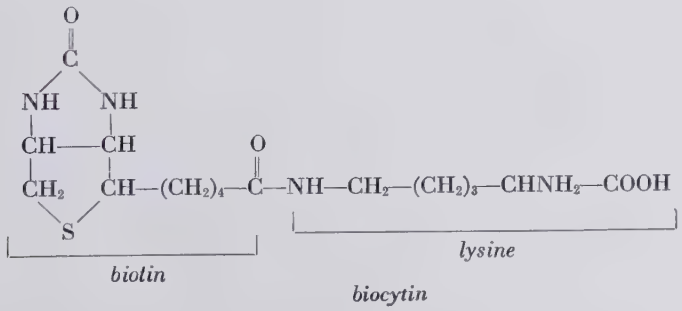
Biosynthesis of Folic Acid Coenzymes



119.



120.



cytin replaces biotin in the nutrition of some, but not all, bacteria.

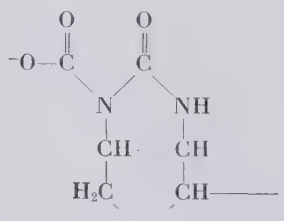
In combination with a specific enzyme protein, biotin reacts with CO_2 to form an enzyme-biotin-CO_2 complex, which, as already discussed, acts as a CO_2 donor in several heterotrophic carbon dioxide fixations. The structure of biotin-CO_2 is shown in **121**.

It is of considerable interest that tetrahydrofolic acid and biotin both function as coenzymes by adding one, two, or three carbon units to active N-groups in the coenzyme molecule.

The biotin requirement of microorganisms, like that of the other B vitamins, is markedly affected by the presence of substances which are synthesized in biotin-dependent reactions. The best example of this dependency is furnished by the inter-relationship of biotin, aspartic acid, and oleic acid in the nutrition of *L. arabinosus*. The bacteria will grow without either aspartic acid or oleic acid if the biotin concentration is high. If aspartic acid is also added, the biotin requirement is cut tenfold; if both aspartic and oleic acids are added, *L. arabinosus* grows without any biotin at all in the medium.

Vitamin B_{12}.[28, 47] This vitamin is a large and complex molecule consisting of (1) a complex ring system closely related to the porphyrins of the cytochromes, chlorophyll, and hemoglobin and synthesized by similar mechanisms, (2) an atom of trivalent cobalt bound to the porphyrin-like structure in much the same fashion as the iron in hemoglobin, (3) a cyanide ion linked to the cobalt ion, (4) a nucleotide containing a substituted benzimidazole as the base, and (5) a molecule of an amino alcohol which is bonded to both the nucleotide phosphorus and a side chain of the central ring system (see **122** on the opposite page). In most natural sources of vitamin B_{12} there also exist a number of B_{12}-like compounds which differ from the true vitamin only in the nature of the nucleotide base. Almost all are biologically inactive.

121.



Biotin-CO_2

Although perhaps the greatest interest in vitamin B_{12} stems from its ability to combat certain human anemias, it is required by many lactobacilli. The use of *L. lactis, L. leichmannii,* and others for microbiological assay (see below) of vitamin B_{12} has greatly aided in isolation and identification of the vitamin. The coenzyme form of vitamin B_{12} required for the conversion of glutamate to β-methylaspartic acid differs from vitamin B_{12} in that (1) the benzimidazole is replaced by adenine and (2) the cyanide ion is replaced by an adenine-containing nucleotide.

Amino Acids. Many bacteria can synthesize all their amino acids themselves (*E. coli*) or can synthesize all but one or two (*Salmonella typhi* requires only tryptophan). In general, the gram-negative organisms are potent amino acid synthesizers and require no preformed amino acids or only a limited number. The ability of gram-positive bacteria to synthesize amino acids is much more limited, particularly among the lactic acid bacteria. *Leuc. mesenteroides* requires, for example, seventeen different amino acids.

Amino acids are required in substantially higher concentrations than are the B vitamins, roughly about 10^{-4} M or about 0.01 mg. per ml. of medium.

Amino acid assimilation. Since gram-positive organisms lack the ability to synthesize many amino acids, they must take them up or assimilate them from the medium. Many gram-positive bacteria, perhaps in compensation for their lack of synthetic powers, have developed efficient mechanisms for assimilating and

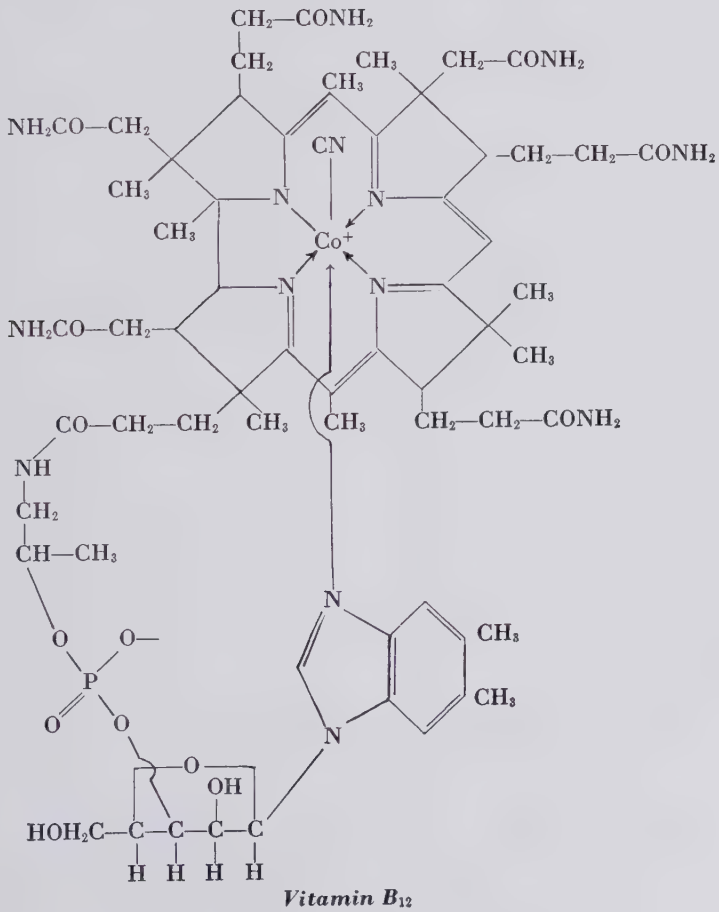
concentrating the amino acids of the medium within their cells. Thus, *Staphylococcus aureus* can assimilate glutamic acid until the intracellular amino acid level is four hundred times that of the external medium. The exact mechanism by which this concentration is achieved is unknown, but it is an active, energy-requiring process. It is of interest that penicillin rather specifically inhibits the active assimilation of amino acids by staphylococci (see Chap. 6). Not all amino acids are concentrated within the cell to the same degree as glutamic acid. Lysine, for example, enters the cell only by diffusion and thus never attains an intracellular concentration higher than that of the medium.

Factors influencing the requirement for amino acids. Although the amino acid requirement of an organism depends ultimately on its genetic composition which in turn defines the range of its synthetic ability, the expression of this requirement varies under different conditions of growth.

(1) *The Effect of Vitamins.* The modification of the requirement for vitamin B_6, biotin, and folic acid by the presence of amino acids in the medium has already been discussed. It is obvious that the reverse also holds true. Thus, pyridoxal may alter the requirement for D-alanine, biotin for aspartic acid, folic acid for methionine, and so on.

(2) *The Effect of Other Amino Acids.* Many amino acids interfere with or *antagonize* the metabolism of other amino

122.



Vitamin B_{12}

acids. For example, *Bacillus anthracis* grows without either leucine or valine. Yet if leucine is added, growth is inhibited, but is restored in the presence of both leucine and valine. These amino acids have similar structures (see structural formulas in **123**), and it is probable that leucine acts as an inhibitor for the synthesis of valine, thus preventing growth until valine is also added. Similarly, tyrosine prevents the synthesis of the closely related phenylalanine by *E. coli*. These are examples of antagonisms between naturally occurring essential metabolites of the same nature as the antagonism between *p*-aminobenzoic acid and the sulfonamides. Many other examples of amino acid antagonisms have been reported.

The amino acid requirement for specific functions, such as the synthesis of an enzyme, may be different from those for growth. *E. coli*, for example, needs exogenous methionine, tyrosine, and asparagine to make arginine decarboxylase.

Peptides as bacterial growth factors. Some lactobacilli have a specific requirement for peptides in addition to free amino acids. However, these peptides are not directly incorporated into bacterial protein. Instead, they seem to be required because the assimilation of the free amino acids corresponding to the peptide is interfered with in some manner. Thus, *L. casei* grows in the absence of vitamin B_6 when supplied with D-alanine and L-alanine peptides, but free L-alanine is ineffective. This is because D-alanine interferes with the utilization of free L-alanine but not of its peptides.

Purines and Pyrimidines. Many bacteria, especially the lactobacilli, are unable to synthesize the purine and pyrimidine bases which they need as constituents of their nucleic acids, nucleotides, etc. In general, the synthetic block appears to be in the formation of the purine or pyrimidine nucleus, and the majority of nitrogen-base–requiring organisms are still capable of some interconversion of the bases. Thus, *L. arabinosus* can utilize either adenine, guanine, hypoxanthine or xanthine as its sole purine source. Other organisms are more exacting and respond much more to one purine than to another.

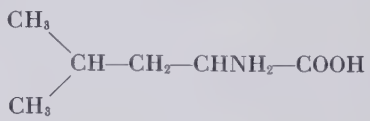
The classic example of a pyrimidine requirement is that of *Staph. aureus* for uracil. It is of interest that uracil is required only for anaerobic growth, not for aerobic.

Purine and pyrimidine requirements are modified by the same type of factors that affect the amino acid requirement. The inter-relation between folic acid and thymine and the purines has already been mentioned, as has the interchangeability of vitamin B_{12} and thymidine and other deoxyribosides in the nutrition of the same bacteria. Antagonisms between the purine bases also affect the purine requirement. In particular, adenine and guanine each interfere with the metabolism of the other in a number of bacteria and yeasts.

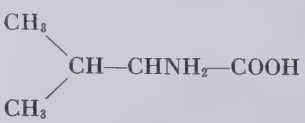
Miscellaneous Organic Growth Factors. Space does not permit consideration of many other growth requirements exhibited by bacteria. Some of the more important ones are listed in the accompanying table.

GROWTH REQUIREMENT	TYPICAL ORGANISM SHOWING REQUIREMENT
Hemin	*Hemophilus influenzae*
Choline	*Diplococcus pneumoniae*
Oleic acid (and other unsaturated fatty acids)	*Corynebacterium diphtheriae*
Lipoic acid	*Lactobacillus casei*
Asparagine	*Streptococcus lactis*
Glutamine	*Streptococcus hemolyticus*
Inositol	*Saccharomyces cerevisiae*
Carbon dioxide	*Brucella abortus*
Mevalonic acid	*Lactobacillus acidophilus*

123.



leucine



valine

It will be noted that most of these compounds have been discussed at some point in the chapter in terms of their role in bacterial metabolism.

The Inorganic Elements. The elementary constituents of the bacterial cell other than the carbon, hydrogen, oxygen, and nitrogen present in organic compounds must also be supplied in an adequate nutritional medium. It is customary to include in bacteriological mediums phosphate, sulfate, potassium, calcium, magnesium, manganous, and ferric ions. The need for these ions in metabolism is obvious. Organic phosphate compounds play a role in almost every phase of intermediary metabolism, and as phospholipids and nucleic acids are a part of the structural framework of the cell. Sulfate, or some form of sulfur, is needed for synthesis of the sulfur amino acids. Potassium, calcium, magnesium, and manganous ions are cofactors or activators in many enzyme systems.

Some organisms also have more specific inorganic requirements. Thus, the diphtheria bacillus gives optimum toxin production with an iron concentration of about 0.00014 mg. per ml. of medium. Higher or lower concentrations inhibit toxin production without affecting growth. Azotobacter specifically requires molybdenum for some phases of nitrogen fixation.

Undoubtedly other inorganic ions also appear in bacterial protoplasm and, therefore, must be present in the medium. However, it is very hard to show clearly that a particular ion is absolutely required for growth. This is because the inorganic ions are universal contaminants of all types of materials, so that it is almost impossible to free mediums of the last traces of an ion. Then, because the inorganic ions are needed only in the most minute amounts, only rarely can it be shown that no growth of an organism occurs except upon addition of the ion to be tested. For example, bacteria which do not require vitamin B_{12}, and are known to synthesize it, should need a supply of cobalt for growth, but such a need has not yet been demonstrated.

Microbiological Assay.[25] An important practical application of the elucidation of the growth factor requirements of bacteria has been the development of methods for quantitative determination of vitamins and amino acids in terms of the growth response of microorganisms.

In principle, the methods of microbiological assay are simple. Taking a specific example, *L. helveticus* is a suitable organism for the assay of riboflavin because it does not grow at all without it, yet grows vigorously upon addition of as little as 0.0001 mg. per ml. to an otherwise adequate medium. An assay medium is selected which is free of riboflavin, but adequate in every other respect. To aliquots of the medium are added several different concentrations of a riboflavin standard over a range in which growth is proportional to the amount of riboflavin added. At the same time, graded amounts of the unknown sample to be assayed are added to other aliquots of the medium. Then, all aliquots are inoculated and incubated, and the growth produced by the unknown is compared with the growth produced by the riboflavin standard. From this comparison, the concentration of riboflavin in the unknown sample may be determined.

In practice, the great complexity of bacterial metabolism makes it necessary to pay careful attention to every detail of the assay procedure in order to obtain accurate, reproducible results. The occurrence of different forms of a growth factor with different growth-promoting activities, and the modification of the response to the growth factor being assayed by other growth factors in the medium, are examples of possible sources of error in microbiological assay.

Nevertheless, the high degree of specificity and sensitivity attainable in microbiological assay have made this technique an invaluable tool in many fields of biological research.

REFERENCES

1. Albersheim, P., and Bonner, J.: Thiamine. In Greenberg, D. M. (ed.): Metabolic Pathways, vol. 2, pp. 617–625. Academic Press, New York, 1961.
2. Asselineau, J., and Lederer, J.: Chemistry and metabolism of bacterial lipides. In Bloch, K.

(ed.): Lipide Metabolism, pp. 337–406. John Wiley and Sons, New York. 1960.
3. Axelrod, B.: Glycolysis. In Greenberg, D. M. (ed.): Metabolic Pathways, vol. 1, pp. 97–128. Academic Press, New York. 1960.
4. Barker, H. A.: Fermentation of nitrogenous organic compounds. In Gunsalus, I. C., and Stanier, R. Y. (eds.): The Bacteria, vol. 2, pp. 151–208. Academic Press, New York. 1961.
5. Bassham, J. A., and Calvin, M.: The Path of Carbon in Photosynthesis. Prentice-Hall, Englewood Cliffs, New Jersey. 1957.
6. Beinert, H.: Flavin coenzymes. In Boyer, P. D., Lardy, H., and Myrbäck, K. (eds.): The Enzymes, vol. 2, pp. 340–415. Academic Press, New York. 2nd ed., 1960.
7. Belozersky, A. N., and Spirin, A. S.: Chemistry of the nucleic acids. In Chargaff, E., and Davidson, J. N. (eds.): The Nucleic Acids, vol. 3, pp. 147–186. Academic Press, New York. 1960.
8. Berg, P.: Specificity in protein synthesis. Ann. Rev. Biochem., 1961, **30:**293–324.
9. Braunstein, A. E.: Pyridoxal phosphate. In Boyer, P. D., Lardy, H., and Myrbäck, K. (eds.): The Enzymes, vol. 2, pp. 113–184. Academic Press, New York. 2nd ed., 1960.
10. Buchanan, J. M.: Biosynthesis of purine nucleotides. In Chargoff, E., and Davidson, J. N. (eds.): The Nucleic Acids, vol. 3, pp. 303–322. Academic Press, New York. 1960.
11. Cheldelin, V. H.: Metabolic Pathways in Microorganisms. John Wiley and Sons, New York. 1961.
12. Cohen, P. P., and Sallach, H. J.: Nitrogen metabolism of amino acids. In Greenberg, D. M. (ed.): Metabolic Pathways, vol. 2, pp. 1–78. Academic Press, New York. 1961.
13. Crosbie, G. W.: Biosynthesis of pyrimidine nucleotides. In Chargaff, E., and Davidson, J. N. (eds.): The Nucleic Acids, vol. 3, pp. 323–348. Academic Press, New York. 1960.
14. Crick, F. H. C., Barnett, L., Brenner, S., and Watts-Tobin, R. J.: General nature of the genetic code for proteins. Nature, 1961, **192:** 1227–1232.
15. Dedonder, R. A.: Glycosidases and transglycosidation. Ann. Rev. Biochem., 1961, **30:**347–382.
16. Delbrück, M., and Stent, G.: On the mechanism of DNA replication. In McElroy, W. D., and Glass, B. (eds.): The Chemical Basis of Heredity, pp. 699–736. Johns Hopkins Press, Baltimore. 1957.
17. Dolin, M. I.: Survey of microbial electron transport mechanisms. In Gunsalus, I. C., and Stanier, R. Y. (eds.): The Bacteria, vol. 2, pp. 319–363. Academic Press, New York. 1961.
18. Dolin, M. I.: Cytochrome-independent electron transport enzymes of bacteria. In Gunsalus, I. C., and Stanier, R. Y. (eds.): The Bacteria, vol. 2, pp. 425–460. Academic Press, New York. 1961.
19. Green, D. E., and Wakil, S. J.: Enzymatic mechanisms of fatty acid oxidation and synthesis. In Bloch, K. (ed.): Lipide Metabolism, pp. 1–40. John Wiley and Sons, New York. 1960.
20. Greenberg, D. M.: Carbon catabolism of amino acids. In Greenberg, D. M. (ed.): Metabolic Pathways, vol. 2, pp. 79–172. Academic Press, New York. 1961.
21. Greenberg, D. M.: Biosynthesis of amino acids and related compounds. In Greenberg, D. M. (ed.): Metabolic Pathways, vol. 2, pp. 174–236. Academic Press, New York. 1961.
22. Gunsalus, I. C., and Shuster, C. W.: Energy-yielding metabolism in bacteria. In Gunsalus, I. C., and Stánier, R. Y. (eds.): The Bacteria, vol. 2, pp. 1–58. Academic Press, New York. 1961.
23. Hassid, W. Z.: Biosynthesis of complex saccharides. In Greenberg, D. M. (ed.): Metabolic Pathways, vol. 1, pp. 251–300. Academic Press, New York. 1960.
24. Heppel, L. A., Singer, M. F., and Hilmoe, R. J.: The mechanism of action of polynucleotide phosphorylase. Ann. N. Y. Acad. Sci., 1959, **81:**635–644.
25. Hutner, S. H., Cury, A., and Baker, H.: Microbiological assays. Anal. Chem., 1958, **30:**849–867.
26. Jacob, F., and Monod, J.: Genetic regulatory mechanisms in the synthesis of proteins. J. Mol. Biol., 1961, **3:**318–356.
27. Jaenicke, L., and Lynen, F.: Coenzyme A. In Boyer, P. D., Lardy, H., and Myrbäck, K. (eds.): The Enzymes, vol. 3, pp. 3–104. Academic Press, New York. 2nd ed., 1960.
28. Jukes, T. H., and Broquist, H. P.: Biogenesis and metabolism of folic acid and vitamin B_{12}. In Greenberg, D. M. (ed.): Metabolic Pathways, vol. 2, pp. 713–740. Academic Press, New York. 1961.
29. Kaplan, N. O.: The pyridine coenzymes. In Boyer, P. D., Lardy, H., and Myrbäck, K. (eds.): The Enzymes, vol. 3, pp. 105–169. Academic Press, New York. 2nd ed., 1960.
30. Kaplan, N. O.: Metabolic pathways involving niacin and its derivatives. In Greenberg, D. M. (ed.): Metabolic Pathways, vol. 2, pp. 627–672. Academic Press, New York. 1961.
31. Kates, M.: Lipolytic enzymes. In Bloch, K. (ed.): Lipide Metabolism, pp. 156–237. John Wiley and Sons, New York. 1960.
32. Kornberg, H. L., and Elsden, S. R.: The metabolism of 2-carbon compounds by microorganisms. In Nord, F. F. (ed.): Advances in Enzymology, vol. 23, pp. 401–470. Interscience, New York. 1961.
33. Krampitz, L. O.: Cyclic mechanisms of terminal oxidation. In Gunsalus, I. C., and Stanier, R. Y. (eds.): The Bacteria, vol. 2, pp. 209–257. Academic Press, New York. 1961.
34. Krampitz, L. O., Suziki, I., and Gruell, G.: Role of thiamin diphosphate in catalysis. Fed. Proc., 1961, **20:**971–979.
35. Krebs, H. A., and Lowenstein, J. M.: The tricarboxylic acid cycle. In Greenberg, D. M. (ed.): Metabolic Pathways, vol. 1, pp. 130–203. Academic Press, New York. 1960.
36. Lees, H.: Energy metabolism in chemolithotropic bacteria. Ann. Rev. Microbiol., 1960, **14:**83–98.
37. Lehman, I. R.: Enzymatic synthesis of desoxyribonucleic acid. Ann. N. Y. Acad. Sci., 1959, **81:**745–756.
38. Moat, A. G., and Friedman, H.: The biosynthesis and interconversion of purines and their derivatives. Bact. Rev., 1960, **24:**309–339.
39. Nason, A.: Enzymatic pathways of nitrate, nitrite and hydroxylamine metabolism. Bact. Rev., 1962, **26:**16–41.
40. Nason, A., and Takahashi, H.: Inorganic nitrogen metabolism. Ann. Rev. Microbiol., 1958, **12:**203–246.

41. Nathans, D., von Ehrenstein, G., Monro, R., and Lipmann, F.: Protein synthesis from aminoacyl-soluble ribonucleic acid. Fed Proc., 1962, **21:**127–133.
42. Nirenberg, M.: Approximation of the genetic code via cell-free protein synthesis directed by template RNA. Fed. Proc., 1963, **22:**51–61.
43. Novelli, G. D.: Protein synthesis in microorganisms. Ann. Rev. Microbiol., 1960, **14:**65–82.
44. Ochoa, S.: Synthetic polynucleotides and the amino acid code. Fed. Proc., 1963, **22:**62–74.
45. Ochoa, S., and Kaziro, Y.: Biotin enzymes. Fed. Proc., 1961, **20:**982–988.
46. Plaut, G. W. E.: The biosynthesis of flavin derivatives. In Greenberg, D. M. (ed.): Metabolic Pathways, vol. 2, pp. 673–712. Academic Press, New York. 1961.
47. Plaut, G. W. E.: Water-soluble vitamins. II. Folic acid, riboflavin, thiamin, vitamin B_{12}. Ann. Rev. Biochem., 1961, **30:**409–446.
48. Quale, J. R.: Metabolism of C_1 compounds in autotrophic and heterotrophic microorganisms. Ann. Rev. Microbiol., 1961, **15:**119–152.
49. Rabinowitz, J.: Folic acid. In Boyer, P. D., Lardy, H., and Myrbäck, K. (eds.): The Enzymes, vol. 2, pp. 185–252. Academic Press, New York. 2nd ed., 1960.
50. Racker, E.: Mechanisms of synthesis of adenosine triphosphate. In Nord, F. F. (ed.): Advances in Enzymology, vol. 23, pp. 323–399. Interscience, New York. 1961.
51 Reed, L. J.: Lipoic acid. In Boyer, P. D., Lardy, H., and Myrbäck, K. (eds.): The Enzymes. 2nd ed. vol. 3, pp. 195–224. Academic Press, New York. 1960.
52. Reichard, P.: The enzymatic synthesis of pyrimidines. In Nord, F. F. (ed.): Advances in Enzymology, vol. 21, pp. 263–294. Academic Press, New York. 1959.
53. Rogers, H. J.: The dissimilation of high molecular weight substances. In Gunsalus, I. C., and Stanier, R. Y. (eds.): The Bacteria, vol. 2, pp. 257–318. Academic Press, New York. 1961.
54. Schulman, M. P.: Purines and pyrimidines. In Greenberg, D. M. (ed.): Metabolic Pathways, vol. 2, pp. 389–457. Academic Press, New York. 1961.
55. Smith, L.: Cytochrome systems in aerobic electron transport. In Gunsalus, I. C., and Stanier, R. Y. (eds.): The Bacteria, vol. 2, pp. 365–396. Academic Press, New York. 1961.
56. Stanier, R. Y.: Photosynthetic mechanisms in bacteria and plants: development of a unitary concept. Bact. Rev., 1961, **25:**1–17.
57. Talalay, P.: Enzymatic mechanisms in steroid metabolism. Physiol. Rev., 1957, **37:**362–389.
58. Volkin, E.: Synthesis and function of the DNA-like RNA. Fed. Proc., 1962, **21:**112–119.
59. Warren, L.: Nucleotides and nucleosides. In Greenberg, D. M. (ed.): Metabolic Pathways, vol. 2, pp. 459–524. Academic Press, New York. 1961.
60. Watson, J. D., and Crick, F. H. C.: Genetical implications of the structure of deoxyribonucleic acid. Nature, 1953, **171:**964–969.
61. Weiss, S. B.: Biosynthesis of ribopolynucleotides. Fed. Proc., 1962, **21:**120–126.
62. Wood, W. A.: Fermentation of carbohydrates and related compounds. In Gunsalus, I. C., and Stanier, R. Y. (eds.): The Bacteria, vol. 2, pp. 59–150. Academic Press, New York. 1961.

Chapter 6

PHYSICAL AGENTS, BACTERICIDAL SUBSTANCES (DISINFECTANTS), AND CHEMOTHERAPEUTIC DRUGS

THE SEPARATION of the environmental factors, that so profoundly affect the life processes of living cells, into two groups, one designated physical and the other chemical, is undoubtedly artificial in many instances. It is often difficult, if not impossible, to differentiate the physical from the chemical; the lethal effect of a germicidal substance, for example, may be a result of a combination of both physical and chemical activity or its mode of action may lie in the borderland of surface and physical chemistry. Nevertheless, such a separation has a certain reality and is useful for purposes of exposition.

Bacteria, like all other living organisms, consist of protoplasm, a delicately balanced, heterogeneous mixture of various substances in colloidal and true solution. The disturbance of this equilibrium through, for instance, the precipitation of constituent protein is incompatible with the continuation of the complex phenomena of life, and the cell dies. On the other hand, the environment, when favorable, not only does not destroy this equilibrium but makes possible its continuation through growth and multiplication of the organisms. Bacteria, like other organisms, are creatures of their environment; under favorable conditions they multiply rapidly, and under unfavorable conditions either die or remain dormant in a viable state until another opportunity for growth presents itself. In general, they are much more resistant to unfavorable circumstances than are most higher forms of life. A part of this resistance is, of course, a result of the ability of some bacteria to form spores which are relatively highly resistant, but the vegetative cells are considerably more resistant than are the cells of multicellular organisms.

The inactivation of viruses tends to be set somewhat apart since they consist of nucleic acid protected, in the vegetative form, by a protein coat whose contribution to viral replication is not altogether clear, and they are not physiologically complete units like the bacteria.[23, 76] Thus bacteria could not survive the treatment with 50 per cent phenol, a good disinfectant, used to extract infectious ribonucleic acid from many RNA-containing viruses, nor are bacteria or vegetative viruses destroyed by treatment with ribonuclease as is infectious RNA.

It is not possible to differentiate sharply between these favorable and unfavorable factors. The congruity of a

given environmental factor with the protoplasmic equilibrium that is life is largely a quantitative rather than a qualitative phenomenon. Although high temperatures destroy bacteria, a certain degree of warmth is essential to their growth. Distilled water is toxic to many microorganisms, yet multiplication takes place only in the presence of adequate amounts of moisture. Even highly active germicidal chemicals often markedly stimulate growth of bacteria, when in sufficiently low concentrations.

PHYSICAL AGENTS[81]

Temperature Relations. Bacteria as a group will thrive under a relatively wide range of temperature conditions. Some of the more hardy, such as *Bacillus subtilis,* will grow throughout the range of 6° to 50° C. Others, such as many of the pathogenic forms, are able to grow over a much narrower range, and some of the more fragile organisms will grow only at body temperature, *i.e.,* 37° C., or very close to this temperature. For all these organisms, however, three temperature limits may be distinguished. There is the *minimum* or lowest temperature at which a given organism will grow, an *optimum* or temperature of most luxuriant growth, and a *maximum,* the highest temperature at which growth can take place. The position of these three points differs greatly among different species of bacteria. In general, those organisms whose natural habitat is soil or water have optimum temperatures of 22° to 28° C., while those which, presumably as a result of adaptation to a parasitic mode of existence, cannot survive outside the animal body, have an optimum of 37° C.

There are, however, bacteria whose optimum temperatures differ considerably from these. Organisms have been found whose optimum temperatures are from 15° to 20° C., and they have been termed *psychrophiles* or cold-loving organisms. Others having optimum temperatures of 55° to 65° C. may be found in the soil and hot springs and are called *thermophiles.* The great majority of bacteria have optimum temperatures which lie between these two extremes and, in this terminology, are designated as *mesophiles.*

The optimum temperature for given species of bacteria is generally considered to be that temperature at which the organisms grow "best." The criteria of bacterial growth have been discussed elsewhere (Chap. 4). So far as the effects of incubation temperatures are concerned, those optimum for growth in terms of rapidity of cell division are not always the same as those optimum for the attainment of maximum numbers of cells per unit volume. A culture grown at a temperature at which cell multiplication is most rapid will not attain as high a peak in numbers as a culture of the same organism growing more slowly at a lower temperature.[94, 98, 99]

Other physiological activities of the cell appear to have optimum temperatures that differ from those which are optimum for multiplication. A given sugar may be fermented to a greater extent, more slowly, when the culture is incubated at a temperature somewhat below that optimum for cell division. Similarly, the anthrax bacillus forms spores most abundantly at 30° to 32° C., while its optimum for vegetative multiplication is 37° C.

The continued growth of bacteria at temperatures somewhat higher than optimum may induce physiological charges of a temporary or permanent character. *Serratia marcescens* (*Chromobacter prodigiosum*), for example, fails to form its characteristic red pigment when incubated at temperatures higher than 30° C., but the change is temporary, for

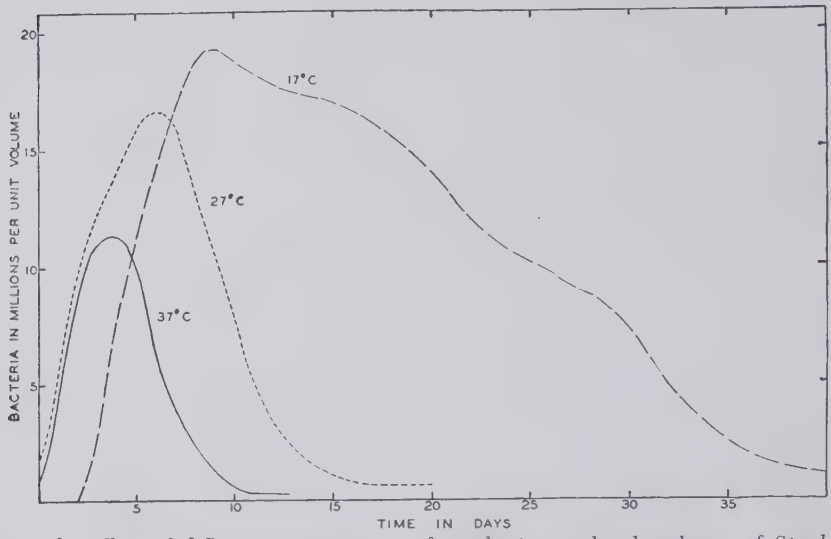


Figure 47. The effect of different temperatures of incubation on broth cultures of *Staphylococcus aureus*. (After Graham-Smith.)

subcultures incubated at lower temperatures form pigment normally regardless of how many transfers have been grown at the higher temperature. The anthrax bacillus, on the other hand, when grown for several transfers at 42° C., loses its ability to form spores and becomes avirulent—a change that appears to be permanent. Incubation or storage of bacterial cultures at temperatures lower than optimum does not result in such qualitative physiological changes; the metabolic activities of the organisms are slowed down and at temperatures below the minimum for growth the bacteria become dormant.

The mechanisms determining the optimum, minimum, and maximum temperatures of bacteria are obscure. In some cases they may be dependent upon other environmental factors. Thus, while many of the thermophilic bacteria are able to grow only at temperatures above 50° C. when in contact with air, they are able under anaerobic conditions to grow at the ordinary incubator temperature (37° C.) or even as low as 34° C. There is evidence too that the maximum growth temperatures of bacteria bear a definite relationship to the minimum temperature of destruction of respiratory enzymes. The enzymes of thermophilic bacteria do not, however, have unusually high inactivation temperatures; rather, thermophilic growth is possible because the rate of synthesis of enzymes and other thermolabile cell constituents exceeds the rate of their destruction at the high environmental temperatures.[2]

The Lethal Effects of Heat. Bacteria are readily killed by heat, and the utilization of heat in one form or another is one of the most convenient means of their destruction. The lethal effects of heat are markedly influenced by the amount of moisture present, and so-called *moist heat* is a much more effective killing agent than dry heat. The resistance of bacteria to moist heat differs somewhat from species to species, the pathogenic forms being in general somewhat less resistant. The resistance of a given species is influenced by two important factors: the ability of the organisms to form spores and the previous history of the culture. Spores are always much more resistant than vegetative forms. Some species when in the spore stage can withstand the temperature of boiling water for upward of 16 hours. The vegetative forms of most bacteria, on the other hand, are killed at 55° to 58° C. by 30 minutes' exposure in the presence of moisture.

The time and temperature of incubation influence, to some extent, the heat resistance of vegetative cells. Actively growing cultures in the logarithmic

phase are generally somewhat less resistant than are cells removed from cultures containing maximum numbers of viable organisms. The temperature of incubation appears to affect thermal resistance somewhat, in that cultures grown at and above the optimum temperature are more resistant than cultures grown at suboptimal temperatures.

The pH of the liquid in which the microorganisms are suspended is of considerable quantitative importance. Deviations from neutrality increase, to a marked degree, the rate at which a given temperature kills bacteria. Acid foods, such as tomatoes, are more readily preserved than foods which have a neutral reaction. Likewise, the addition of sodium carbonate to water in which surgical instruments are boiled increases the efficiency of the heat and at the same time tends to reduce rusting.

The death of an organism from heat is determined not only by the temperature reached but also by the time of exposure. The tubercle bacillus, for example, is killed by 30 minutes' exposure at 58° C., 20 minutes' at 59° C. and 2 minutes' at 65° C. One may, therefore, determine the *thermal death point* of a given bacterium by exposure to varied degrees of heat for a constant time, or, similarly, a *thermal death time* may be determined by holding the temperature constant and varying the time of exposure. Both are useful, the latter possibly somewhat more so.

The effect of heat seems to be injurious even when bacteria are not killed, since the cells that have been heated appear to require a longer period of germination. Exposure to sublethal heat prolongs the latent period preceding cell division without affecting the rate of regeneration of the respiratory function, with no evidence of a period of "recovery" distinct from growth. On the other hand, sublethal heating of bacterial spores often has the effect of accelerating germination.

Sterilization by heat. The application of moist heat to the destruction of bacteria may take several forms. Sterilization by means of steam under pressure is the most efficient of these, because it makes possible temperatures higher than 100° C. in the presence of moisture. At 15 pounds' pressure, for example, the temperature will be 121.3° C., and at 20 pounds', 126.2° C. As a rule, exposure to 120° C. for 15 minutes suffices for the complete destruction of both vegetative and spore forms of bacteria, although rarely highly resistant spores may be found that are not killed by this exposure. Boiling, *i.e.*, exposure to 100° C., suffices to kill all vegetative forms of bacteria within a few minutes, but sterilization is not effected, for the spores of many of the saprophytic bacilli are not destroyed by boiling for many hours. Since very few of the pathogenic bacteria form spores, boiling contaminated water for a few minutes renders it safe for drinking purposes, and the boiling of surgical instruments generally suffices to kill the vegetative cells of pathogenic bacteria, but not the spores of spore-forming pathogens such as those of the tetanus and gaseous gangrene bacilli.

Temperatures as high as 100° C. are not necessary for the destruction of vegetative forms of bacteria, since most of them are killed at 55° to 58° C. if exposed for 10 to 30 minutes. In the preparation of vaccines, suspensions of bacteria are usually heated to 60° C. for 30 minutes to one hour (to allow an adequate margin of safety). The tubercle bacillus, *Brucella abortus,* and other pathogenic organisms occurring in milk are killed by the process of pasteurization, *i.e.*, heating to 142° to 145° F. for 30 minutes.

Dry heat is much less effective as a germicide than moist heat. Temperatures of 160° to 170° C. must be maintained for two to three hours in order to ensure complete destruction.

Sterilization by heating is a common and useful procedure, and the nature of the material to be sterilized determines whether moist heat or dry heat is used. Bacteriological mediums, solutions, etc., are sterilized by steam under pressure in an autoclave provided that the contained substances are heat-stable, while laboratory glassware, syringes and hypodermic needles, etc., are usually sterilized by dry

heat in a thermostatically controlled oven (see Chap. 2). The temperature is ordinarily kept constant, 120° C. and 170° C. respectively, but the time required for sterilization depends upon the volume of liquid, how closely the containers are packed, and similar factors that affect penetration of heat to effective sterilizing temperatures.

The destructive effect of high temperatures on bacteria is apparently associated with the coagulation of constituent protein. The rate of heat coagulation of protein in solution closely parallels the rate of destruction of bacteria by hot water. Furthermore, the effect of the water content of egg albumin on the temperatures necessary for coagulation is similar to the observed relative efficiency of moist and dry heat in the killing of bacteria.*

Cold. In general, cold has a preservative effect on microorganisms in that, as the temperature is lowered from the optima for growth processes and other physiological activities, the metabolic rate is reduced. The temperature coefficient is substantially the same as that of other living organisms, *i.e.*, the metabolic rate is reduced by approximately half for each 10° C. drop in temperature over the relatively narrow range in which the effect is demonstrable. Thus, microorganisms grow and metabolize rapidly at incubator temperatures, but tend to persist in a semidormant state at usual refrigerator temperatures (2 to 8° C.), and storage in the refrigerator is a common method of preserving bacterial cultures over a limited time.

The effect of chilling is not invariably preservative. Some microorganisms, such as the influenza virus, are still inactivated sufficiently rapidly at refrigerator temperatures that they may be preserved for only a few hours. Still other microorganisms, notably the gonococcus and meningococcus, tend to die out even more rapidly at refrigerator than at incubator temperatures.

Extreme low temperatures. It is commonly said that microorganisms survive freezing and extremely low temperatures such as those of liquid air (—190° C.) and liquid hydrogen (—250° C.). The effect of exposure to, and storage at, temperatures below the freezing point of water is undoubtedly injurious to a greater or lesser extent, but when some, even relatively few, of the millions of individual cells present are found to be viable, the microorganism is said to "survive" the treatment. Many, perhaps very many, are killed as a consequence of the operation of lethal factors, and the significant variables are the rate of freezing, the temperature at which the frozen cells are stored, and the rate at which they are thawed.[66]

When the cells are frozen slowly there is a preferential formation of extracellular crystal nuclei, and their growth produces an increase in extracellular osmotic pressure, withdrawing water from the cell which is added to the growing crystals. The freezing point of the remaining water is depressed by the increasing concentration of soluble substances as the temperature is reduced; the unfrozen water decreases from as much as 50 per cent of the total at the freezing point to less than 10 per cent at —10° C. When the freezing is extremely rapid, the tendency to extracellular crystallization diminshes and intracellular crystallization occurs. In the first instance two lethal effects are operative, the one that of high electrolyte concentration, and the other dehydration denaturation. Very rapid freezing minimizes the first effect, but not the second.

The lethal effects are time and temperature dependent, declining exponentially with reduction in temperature and extrapolating to zero at the glassy transformation temperature (—130° C.). There is a third potentially lethal factor affecting material that has been frozen sufficiently rapidly to give intracellular ice crystals; that of growth of the intracellular crystals which is also di-

* Egg albumin in aqueous solution is coagulated at 56° C.; with 25 per cent water content at 74° to 80° C.; with 18 per cent water content at 80° to 90° C.; with 6 per cent water content at 145° C. Anhydrous egg albumin may be heated to 170° C. without coagulation.

rectly and exponentially related to temperature. The lethal effects of these factors are also species-dependent in that some kinds of microorganisms are less affected by them than others.

In practice, storage at a temperature of —50° C. or less is required for prolonged viability. For example, the cholera vibrio, one of the more delicate bacteria in this respect, dies off within a few hours when stored as a frozen suspension at —25° to —30° C., but remains viable for at least two years at —65° C. So the ordinary "deep freeze" is not cold enough for storage of frozen bacterial cultures, but a dry-ice refrigerator, or its equivalent, is.

The sequence of events occurring in the course of freezing is reversed in the thawing process, with the rates of denaturation and crystal growth increasing exponentially as the temperature rises. The process is, in fact, more rigorous than that of freezing because of the kinetics of heat exchange, and rapid thawing is required to minimize lethal effects. It is obvious that the effects of these three phases of the process—freezing, storage, and thawing—cannot be separated from one another but may be evaluated only relatively when two are held constant and the third varied.

Drying. The vegetative forms of most bacteria are killed by drying in air, although the different species exhibit pronounced differences in their resistance. The tubercle bacillus is one of the more resistant and the cholera vibrio one of the more sensitive to drying. In general, the capsulated organisms are more resistant than the noncapsulated forms. Spores are quite resistant to drying; the spores of the anthrax bacillus, for example, will germinate after remaining in a dry condition for 10 years or more. The resistance of the pathogenic forms causing disease of the upper respiratory tract is of particular interest in connection with air-borne infection, for the length of time a droplet remains infective is a function, primarily, of the resistance of the particular microorganism to drying.[25] The observed lethal effect of suspension of microorganisms in air is largely a function of relative humidity, and it has been found that maximal death occurs at about 50 per cent relative humidity. This is apparently a consequence of the amount of water retained in the droplets; at very low humidities the rapid desiccation renders the organism resistant to the toxic action of soluble substances concentrated by the partial drying, while at high humidities sufficient moisture is retained to keep such substances from reaching toxic concentrations.

Freeze-drying.[29, 111] The resistance of a given organism to drying is apparently determined to some extent by the rapidity of the drying process and the temperature at which the organisms are dried and stored. If bacteria are frozen rapidly, as with dry ice and alcohol or one of the glycols, dried from the frozen state (the lyophile process), and the container or ampoule evacuated and sealed, they remain viable with antigenicity and virulence unimpaired for months and years when stored in the refrigerator. Most of the bacteria are killed in the process, with less than 1 per cent surviving,[22] and it is these few survivors that grow on subculture. Probably the most important single factor in survival is the menstruum in which the bacteria are suspended. Poor results are usually obtained with ordinary broths, and the addition of normal serum, lactose, and other ingredients contributes to survival.[27] Survival has been of particular interest in the preparation of BCG vaccine in a stable dry form; the inclusion of sodium glutamate apparently contributes considerably to the survival of this microorganism (Chap. 31).[31, 86]

Radiation.[58, 59, 67, 71, 72, 77, 91, 110] There is little biological effect of visible light on non-chlorophyll-containing microorganisms except in the presence of certain dyes which increase absorption of light, and energy, to produce inactivation. This appears to be due in large part to the formation of peroxides and is inhibited in the presence of catalase. The phenomenon is called photodynamic sensitization.

Beginning in the ultraviolet region, the

biological effects of radiation become pronounced, and the well-known bactericidal activity of direct sunlight is due to its content of ultraviolet light. The effects of ionizing radiation, x-rays, and γ-rays (short x-rays) being the most commonly used, are even more marked.

With relatively large doses of radiation the microorganism is inactivated in that it will no longer reproduce, or, in the case of pathogens, produce infection, although overt evidences of viability such as respiration rate are not affected appreciably. With smaller doses, not sufficient to inactivate, the genetic mechanisms of irradiated microorganisms become unstable, and the mutation rate is increased from the normal spontaneous rate of perhaps 10^{-8} to as much as 10^{-3}. Similar doses applied to lysogenic bacteria activate the latent infection with provirus so that complete virus is produced and the host cells are lysed as described elsewhere (Chap. 4).

Both ultraviolet and ionizing radiation are electromagnetic waves emitted in quanta, but they differ in the way in which biological effects are produced because they are absorbed differently, and because their quanta carry greatly different amounts of energy.

Ultraviolet radiation. Absorption in the region between 2500 Å and 3000 Å is due in part to the aromatic amino acids and cystine, with the peak at 2800 Å attributable to tryptophan and tyrosine. The peak absorption by nucleic acids, *i.e.*, the purine and pyrimidine rings, occurs at around 2600 Å. Nucleic acids absorb much more strongly than proteins, and twenty-five to fifty times more energy is absorbed per unit weight by the former than by the latter. It is more than coincidence that the region of maximal absorption in the ultraviolet is also that of maximal antimicrobial activity.

At 2537 Å, the most effective and commonly used wave length, a quantum of ultraviolet radiation has an energy of about 5 ev. This is not sufficient to eject an electron to form an ion, a process which requires an average of 30 ev in biological material, but is enough to raise a constituent electron to a higher state of energy and thus produce excitation rather than ionization. Modification of molecules occurs by the breaking of bonds (for example a covalent bond may be broken by about 4 ev), but since such events occur at random in the sense that they do not necessarily affect essential functional mechanisms, the effects of ultraviolet radiation are less than those of ionizing radiation. The surrounding medium is affected also, and the formation of peroxides contributes to the antimicrobial effects of irradiation. In total, about 100 times as much energy is required to inactivate microorganisms by ultraviolet radiation as by x-radiation, and it is calculated that about 4 million quanta are necessary to inactivate a colon bacillus.

The effects of ultraviolet radiation are not completely established immediately, and for some time, even as much as several hours, they may be modified by exposure of the microorganisms to visible light. This phenomenon is that of photoreactivation.[15] The mechanisms involved are far from clear, and two kinds of hypotheses are current. The exposure to visible light may, in some obscure way, repair damage caused by the inactivating radiation, or it may provide a possible alternative mechanism to replace that which was damaged. Reactivation may also occur following short exposure to heat (45° C.) or the addition of substances such as catalase, peroxidase, or some organic substances.

Ionizing radiation. Ionizing radiation includes α-rays (nuclei of helium atoms), β-rays (fast-moving electrons), γ-rays, and x-rays. As ionizing radiation passes through a microorganism, it collides with successive atoms to eject electrons and so create columnar zones of intense ionization. Secondary ionization, produced by ejected electrons along different paths (δ-radiation) may be of quantitative significance, especially with α-radiation.

The resistance of microorganisms, both bacteria and viruses, is relatively high, 10^6 rad* or more being required for

* A rad is defined as the amount of radiation required for the absorption of 100 ergs of energy per gram of substance having unit density.

99 per cent inactivation. There is reason to believe that radiation effects may be a consequence of a direct hit of key substances within the cell, and their destruction or alteration by the intense ionization. This is the target theory, but it is not fully consistent with radiation protection.[42]

The effect of radiation are modified in various ways. The oxygen effect, *i.e.*, the reduced radiosensitivity of a microorganism when oxygen is displaced with an inert gas or removed by metabolism, is well known. Similarly, certain compounds, especially those containing sulfhydryl groups, give excellent protection against radiation damage.

Radiation damage. The effects produced are basically biochemical in nature and appear to revolve to a considerable extent about injury to the genetic mechanisms, including the blocking of DNA synthesis. Other synthetic reactions, notably the formation of adaptive enzymes (Chap. 7), are inhibited also. The processes of cell division appear to be affected more readily than at least some synthetic processes, and inactivated rod-shaped bacteria, for example, continue to grow for a time in filamentous form but do not reproduce. The marked increase in mutation rate resulting from irradiation in doses less than those required for inactivation has been taken to suggest that inactivating doses may give rise to lethal mutations which prevent subsequent multiplication of the irradiated cells.

Radiation-pasteurization.[3, 69] Inactivation of microorganisms by irradiation has some practical application in the preparation of vaccines and possibly also in the preservation of food. There is some reason to believe that vaccines prepared by radiation-inactivation of pathogenic microorganisms, such as dysentery bacilli and poliovirus, are superior immunizing agents because this kind of inactivation produces less change in significant antigenic substances than treatment with heat or disinfectants such as formaldehyde. Ultraviolet radiation has been used for this purpose but, because its lack of penetration poses some technical problems in the application of uniformly inactivating but not excessive doses, has not been widely used.

The application of irradiation to the preservation of foods has been of considerable interest. Radiation having high penetration is required, and of the several types, x-ray and γ-ray have practical applicability in this respect. X-rays may be generated in the conventional ways, and Co^{60} can be used as a source of γ-radiation. The spores of *Clostridium botulinum*, routinely used for evaluating many food preservation procedures, are relatively resistant to radiation, and it has been found, for example, that 10^6 to 10^7 rad is required for the destruction of the spores in meat to the acceptable standard of efficacy. While there is no residual radioactivity of any consequence, undesirable flavors are often produced which can be only partially eliminated by devices such as the incorporation of acceptable free radical acceptors. At lower dosages partial destruction is achieved which may have some utility in supplementing other procedures such as refrigeration.

Electrophoresis. Microorganisms are apparently unaffected by the passage of electric currents through their suspensions, provided that the effects of heating and acidity and alkalinity in the vicinity of the electrodes are controlled. They do, however, carry a charge that is a composite of those of the amphoteric substances comprising them, and at neutrality they are negatively charged, with an iso-electric point at pH 3 to 4. At alkalinities above the iso-electric point they move in the electric field toward the cathode, and this is known in the older literature as cataphoresis rather than electrophoresis. Bacteria are too large to move freely in a supporting medium such as paper, but certain of the viruses are separable by paper electrophoresis.

Cell Disintegration. The bacterial cell may be broken up by physical means in a number of ways. The cells may, for example, be dried, commonly with acetone, or frozen, and ground with alumina. The cell structure may also be

broken down by the grinding action developed in shaking vigorously with small glass beads or similar particles. This is the principle of the Mickle disintegrator which shakes a mixture of bacterial suspension and small glass beads rapidly at the ends of a tuning fork. Or bacteria may be broken up by subjecting them to sudden changes in pressures of the order of 500 to 600 atmospheres, by sudden application or release of the pressure, but they are killed without disruption by the application of very high pressures, such as 10,000 atmospheres, which also denature proteins. Methods such as these split the cell wall, allowing the constituents of the cell to escape, and are used for the preparation of cell walls as well as for the study of intracellular enzyme activity, etc.

Still another method of breaking up the cell is treatment with sonic or ultrasonic vibrations. Sonic vibration at 9 to 10 kc. breaks up fragile bacteria, such as the cholera vibrio, in a few minutes, but others, such as lactobacilli, require longer treatment. Ultrasonic vibration at 30 to 100 kc. with reasonably large power input, perhaps 400 w., breaks up the cells much more rapidly. In either case there is a marked tendency to fragment the cell wall also, and some degree of denaturation of enzymatic activity often occurs.

Such physical destruction of the bacterial cell is, of course, lethal in that the fragmented cells are killed, and viable cells are rapidly reduced in number, but complete sterilization is extremely difficult to attain by such methods.

DISINFECTANTS[74, 82, 104, 110, 128]

Many kinds of substances have a deleterious effect on microorganisms. Antimicrobial substances have two kinds of activity, the one bactericidal germicidal, or virucidal, and concerned with the killing of microorganisms, and the other bacteriostatic or growth-inhibiting. The line of demarcation between the two kinds of effects is not a sharp one in that a bactericidal substance may inhibit growth rather than kill in low concentrations or when the period of exposure is limited. It is quite generally observed also that concentrations just below those at which an antimicrobial effect becomes apparent markedly stimulate bacterial growth.

In a general way, a substance is regarded as primarily bacteriostatic when the range of concentration over which inhibition of growth occurs is a relatively wide one, and primarily bactericidal when it is narrow. Similarly, when the rate of killing is slow, the exposed microorganisms may survive, in diminishing numbers, for a considerable time without growth, and, for these survivors, the antimicrobial activity is growth-inhibiting. Conversely, when the rate of killing is rapid, and survivors are detectable for only a short time, the activity is regarded as primarily lethal. The distinction between lethal and growth-inhibiting activity is, therefore, twofold in that it is dependent upon both concentration and rate effects and upon their interrelationship. So the distinction is one of degree rather than kind and is clear only at extremes.

The terms antiseptic and disinfectant are applied to describe antimicrobial activity. Etymologically, both imply the effective removal of pathogenic microorganisms, but by usage they have come to have somewhat different meanings. An antiseptic is a substance that has sufficient antimicrobial activity to interfere with the development of infection, but which is nontoxic when applied superficially to living tissue. A disinfectant is a more potent substance of marked antimicrobial activity which destroys all, or nearly all, pathogenic microorganisms but, because of associated toxicity, is applicable only to inanimate material. In each case the terms must be

defined with respect to the microorganism against which the substance is tested or expected to be used, for a given substance may be an effective disinfectant for one kind of microorganism but not another. The term disinfection is also broadened to cover the process of destruction of pathogenic bacteria by either chemical or physical means.

Antimicrobial activity is a property of a wide variety of substances, both inorganic and organic in nature, and the exploitation of combinations of antimicrobial activity and toxicity and/or corrosive properties is a matter of considerable practical importance. Those having sufficiently low toxicity to allow them to be injected parenterally into the diseased host to reach and maintain effective antimicrobial concentrations in the tissues are the chemotherapeutic drugs, and are discussed in the following section, while those that are useful as disinfectants and antiseptics are considered here.

Inorganic Compounds. A number of inorganic substances have antimicrobial activity because of the toxicity to microorganisms of ions into which they dissociate, or because of their activity as oxidizing agents to bring about some degree of cold combustion of cell substance.

Acids and alkalis. Both strong acids and strong alkalis, *i.e.*, those that are highly dissociated, exert a marked bactericidal effect. The lethal activity of the mineral acids is associated with, and proportional to, the degree of their dissociation, but that of the organic acids appears to be an effect of the whole molecule, for the degree of dissociation is, as a rule, not great. The disinfectant action of alkalis such as sodium hydroxide is likewise proportional to the degree of dissociation. The germicidal activity of the hydroxides of the alkaline earths is, however, greater than can be accounted for on the basis of dissociation, for the metallic ion is often toxic in itself. Both acids and alkalis, in too low a concentration to kill bacteria rapidly, often enhance the activity of other disinfecting agents. For example, the germicidal activity of many salts is greater in the presence of acid or alkali, and, as noted above, bacteria are killed much more rapidly by heat in the presence of dilute acid or alkali than at neutrality.

The relation of bacterial growth to the acidity or alkalinity of culture mediums has been discussed elsewhere. Concentrations of hydrogen or hydroxyl ions compatible with growth are very low, of the order of 10^{-4} to 10^{-9} mols of hydrogen ions per liter. Almost all bacteria will grow at pH 7.0 (1×10^{-7} mols hydrogen ions per liter) but grow best at an optimum which varies from species to species. The minimum and maximum limits between which growth takes place likewise vary widely with species. Certain organisms such as the lactobacilli and *Streptococcus lactis* are termed *aciduric* organisms because they are able to grow profusely at pH 4.0 or less. Perhaps the most acid-resistant organism known is *Thiobacillus thiooxidans.* This organism accumulates sulfate in its cultures as an end product of the oxidation of sulfur and continues to grow even in the presence of N/10 sulfuric acid (Chap. 5).

Salts. Salts have, in general, two effects on bacteria. In very low concentrations they markedly stimulate growth and at higher concentrations they become toxic. The particular concentrations at which these effects are apparent are dependent upon the degree of dissociation of the salt, the nature of the anion and the valency and molecular weight of the metallic ion. In general, the bivalent cations are more toxic than the monovalent cations, and the salts of the heavier metals are more toxic than those of the lighter metals. There is, however, no precise quantitative relation in either case.

The most active of the heavy metals are mercury, silver, and copper. Mercuric chloride is highly active in 0.1 per cent aqueous solution but the activity is bacteriostatic in a sense in that the treated cells may be "revived" by removal of the mercuric ion with hydrogen sulfide. In general, relatively few mercury compounds have antibacterial ac-

tivity, and those which do as a result of the presence of mercuric ion are primarily bacteriostatic rather than bactericidal. The silver salts, such as silver nitrate, although somewhat less active, are still highly efficient germicides. Copper salts are still less active but are highly efficient in the destruction of algae and other chlorophyll-containing organisms. Dissociation of these salts is intimately related to their disinfectant properties. A solution of mercuric chloride in absolute alcohol has substantially no antibacterial activity, but if water is added the activity of the solution increases in proportion to the amount of water added. Because of the importance of ionization, a comparison of the bactericidal power of the various metallic salts on the basis of percentage solution is misleading; equimolecular solutions must be used and the ionization constants taken into consideration. The antimicrobial activity of the heavy metal salts is a result of the affinity of the cations for protein material; when the constituent protein of a bacterial cell is precipitated as an insoluble proteinate, the cell dies. Other factors appear to be involved also, however. It has been observed that inorganic metallic salts which are only slightly bactericidal individually may become markedly active when mixed to produce an oxidation-reduction system.

The oligodynamic action of metals is possibly a result of the solution of the metal to form salts. The destruction of bacteria in contact with or in proximity to a piece of metal is the basis of some methods of disinfection of water. Water may be sterilized by allowing it to seep through a layer of silver-coated sand, and water containing colloidal silver in amounts sufficiently small to defy chemical detection, by calculation about 40 γ per ml., is markedly bactericidal. Such colloidal solutions are prepared by sputtering silver electrodes in water—the so-called catadyn process.

The other cations are less toxic than mercury and silver, but even sodium and potassium are toxic to bacteria in sufficiently high concentrations (*ca.* 2 molar). It is of some interest that the arrangement of cations in an order of their toxicity—mercury and silver at one end and sodium and potassium at the other, with others falling in order in between—corresponds closely to the Hofmeister series and the lyotropic series of Freundlich, in which cations are arranged in the order of their effects on physical properties of proteins, such as coagulation, solubility, and viscosity. The toxicity of cations as manifested in solutions containing a single salt may often be neutralized by the presence, in the proper proportion, of another cation. This phenomenon, known as the antagonistic effect of salts, has led to the concept and preparation of so-called balanced solutions such as Ringer's solution, Locke's solution, and others. Salts not only modify or enhance the toxic qualities of other salts but also exert similar effects on disinfectant compounds of widely different constitution. Sodium chloride, for example, markedly enhances the germicidal qualities of phenol for anthrax spores when present in sufficiently high concentrations.

The part which anions play in the growth and destruction of bacteria is less well known. Some, particularly those containing sulfur, carbon, nitrogen, or oxygen, may serve as sources of these elements and of energy, but in appropriate concentrations many are toxic for bacteria.

Oxidizing agents. Other salts, such as potassium permanganate and the sodium and calcium salts of hypochlorous acid (HOCl), show marked bactericidal activity owing to their properties as oxidizing agents. Mol for mol, hypochlorous acid is one of the most powerful germicides known, and its calcium salt (commonly known as bleaching powder) has a wide use in the treatment of private and small municipal water supplies. Hypochlorous acid reacts with organic compounds containing an amide group with the formation of compounds known as chloramines. These compounds show strong disinfectant properties which are apparently associated with the presence of the =NCl group. Two of these, chloramine-T and

dichloramine-T, were used with considerable success in the disinfection of deep wounds in World War I. Similar compounds such as the chlorates and perchlorates likewise are bactericidal. The high bactericidal activity of chlorine dioxide has been of considerable interest in connection with its use in the treatment of water supplies, since it is relatively unaffected by alkalinity and has other advantages over hypochlorites and chlorine for this purpose. The halogens, chlorine, bromine, and iodine, are also potent germicides but, in contrast to the heavy metals, in inverse order to their atomic weights. Liquid chlorine is widely used in the treatment of water supplies, and iodine in the form of its tincture is an efficient skin disinfectant. Bromine has been used occasionally as a disinfectant for swimming pool water. Fluorine compounds are seldom used as disinfectants, although sodium fluoride is toxic to some bacteria, presumably owing to its interference with their oxidative mechanisms. Both hydrogen peroxide and ozone are bactericidal, but the former is rapidly decomposed by tissue catalase and has little penetrating power when applied to wounds and abrasions.

Organic Compounds. A variety of organic compounds have antimicrobial activity and are useful as antiseptics and disinfectants. The more important are certain metal-organic compounds, phenols and related compounds, soaps and alkyl sulfate detergents, quaternary ammonium compounds, and a few alcohols and ethers.

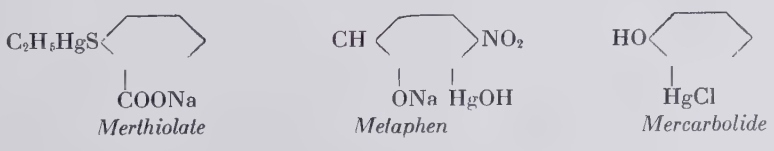
Metal-organic compounds. More or less successful attempts have been made to utilize the germicidal activity of mercury and silver through the preparation of organic compounds which, while having disinfectant properties, are not markedly toxic to body tissue. These include Metaphen, Merthiolate, Mercurochrome (dibrom-oxymercurifluorescein), Mercarbolide, Merphenyl nitrate, Mertoxol (acetoxymercuri-2-ethylhexyl-phenolsulfonic acid), and Meroxyl (sodium salt of 2-4-dihydroxymercury benzophenone-2′-sulfonic acid), together with a series of silver-protein compounds such as Argyrol, Protargol, Argonin, and the like (1). The organic compounds of mercury are generally used as skin disinfectants, though they are of doubtful value, and, in dilute solution, as preservatives. Merthiolate, for example, in 1:10,000 concentration is an excellent and widely accepted preservative for antiserums and similar biologicals. The silver compounds have utility in the treatment of infections of the mucous membranes.

Phenols. Phenol and its derivatives are among the most useful of the antibacterial organic compounds. Phenol itself destroys the vegetative cells of bacteria rapidly, and spores more slowly, in 5 per cent aqueous solution, and its activity is not seriously reduced in the presence of organic matter. It is used as the standard of comparison for other disinfectants, particularly those of similar chemical structure.

The activity of phenol is enhanced by substitution in the ring. The methyl phenols, ortho, meta, and para cresols, and halogenated phenols have greater activity than the parent compound. Resorcinol, hydroxyphenol, however, is only mildly bactericidal. In general, the substitution of aliphatic side chains in both phenol and hydroxyphenol increases antibacterial activity in direct proportion to the length of the side chain, but solubility in water is decreased to limit the practical value of such compounds.[4, 70]

The bisphenols have become the most useful of the phenolic disinfectants because of their relatively high bacteriostatic and fungistatic properties and rel-

1.



atively low toxicity. As indicated by the name, these compounds consist of two phenol rings attached carbon-to-carbon or through oxygen, sulfur, or alkalene, especially methylene, groups. The position of the linkage with respect to the phenolic hydroxyl group is an important factor, and the nomenclature of these compounds becomes involved because the carbon numbered 1 may be that to which the hydroxyl group is attached or that at which the linkage occurs. When the compound is symmetrical either may be used, but only the latter nomenclature may be used when it is asymmetrical.

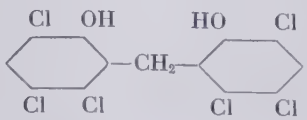
The most important of these compounds are orthohydroxydiphenyl and the chlorinated methylene and sulfur compounds. Orthohydroxydiphenyl is marketed as O'Syl and Lysol, the latter containing cresylic acid also. There are three common chlorophenes. The simplest is dichlorophene (G-4) in which the methylene linkage is ortho to the hydroxy groups and the chlorines in the para positions. Depending upon the system of nomenclature this compound is 2,2′-methylenebis[4-chlorophenol], bis[5-chloro-2-hydroxyphenyl]methane, or 2,2′-dihydroxy-5,5′-dichlorophenylmethane. The other two are tetrachlorophene (G-5) containing two additional chlorines, one on each ring ortho to the hydroxyl group, and hexachlorophene (G-11). The thio bisphenols in common use are bithionol (G-5-S) which corresponds to tetrachlorophene in that it contains four chlorines, and G-11-S which corresponds to hexachlorophene (2). Hexachlorophene has the unusual and useful property of retaining substantially all its antibacterial activity when incorporated into soaps. These compounds are relatively insoluble in water, but are soluble in dilute alkali and in many organic solvents.

These compounds differ from phenol in their activity in that, while they are somewhat more active than phenol under the conditions of the phenol coefficient test (see below), more prolonged exposure is required for maximal bactericidal activity, and they are bacteriostatic in high dilutions. Dichlorophene, for example, gives phenol coefficients of 20 to 40 against staphylococcus and 50 to 75 against the typhoid bacillus, and hexachlorophene 25 to 50 against the former test organism and 5 to 10 against the latter. Dichlorophene, however, inhibits the growth of staphylococcus in dilutions of 1:500,000, and hexachlorophene in dilutions as high as 1:2,500,000.

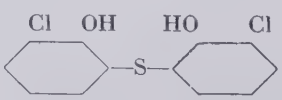
Detergents. The surface-active, detergent substances fall into three groups (3). The anionic compounds are soaps, sodium and potassium salts of higher fatty acids, and the alkyl sulfates such as sodium lauryl sulfate. The quaternary ammonium compounds are cationic substances, and the non-ionic group includes polyethers and polyglycerol esters. Certain of these substances have antibacterial activity.

The antimicrobial activity of soaps is limited, and they cannot be regarded as effective antiseptics or disinfectants. They are useful agents for the mechanical removal of bacteria from the skin by emulsification of lipoidal secretions in which microorganisms are embedded Thus the number of bacteria present on the skin is markedly, but temporarily, reduced by washing with soap. An appreciable antibacterial activity may be given soaps by combining them with disinfectant substances, commonly coal tar products such as cresols and related compounds, but, in general, the activity

2.



hexachlorophene



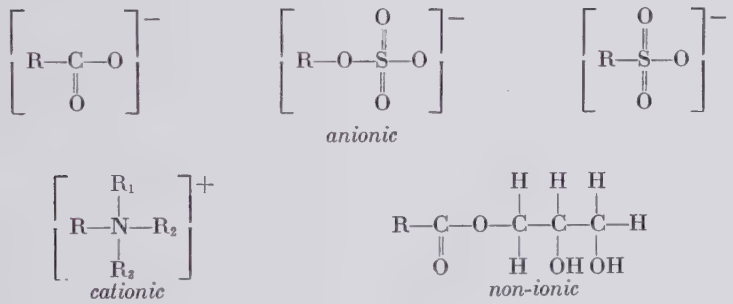
bithionol

of the incorporated substance is reduced in admixture with soap. Hexachlorophene is, as noted above, a significant exception to this general rule. The use of hexachlorophene-containing soaps results not only in an immediate reduction of the numbers of bacteria present on the skin, but the bacteriostatic activity of the residual hexachlorophene significantly inhibits the growth of bacteria on the skin.[41]

Some of the alkyl sulfates have more antibacterial activity than soaps, inhibiting growth in relatively high (0.1 per cent) concentrations. Those which are active are markedly selective, affecting gram-positive bacteria but not gram-negative bacteria.

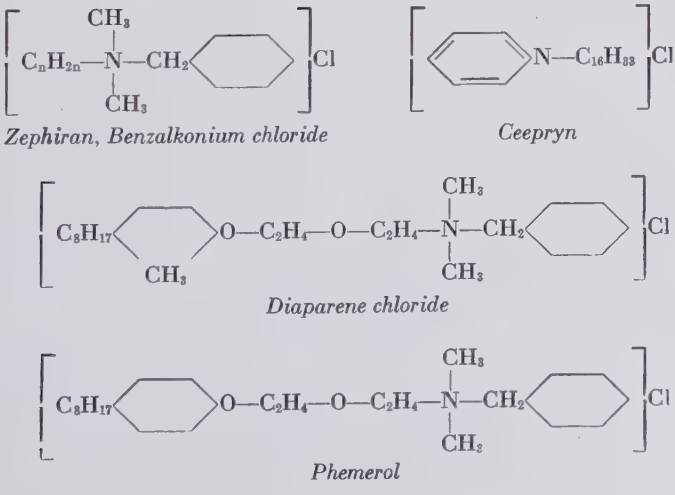
The quaternary ammonium compounds, often called "quats," are a group of amines containing pentavalent nitrogen and may be regarded as derivatives of ammonium chloride in which various radicals are substituted for the hydrogens. Ordinarily one is a long-chain (C_8 to C_{18}) alkyl group, and the others smaller alkyl groups, phenyl groups, etc. Very many, perhaps a thousand, of these compounds have been synthesized, and several, including Zephiran (alkyldimethylbenzylammonium chloride), Ceepryn (cetylpyridinium chloride), Phemerol (diisobutylphenoxyethoxyethyldimethylbenzyl ammonium chloride), and Diaparene (diisobutylcresoxyethoxyethyldimethylbenzyl ammonium chloride) have gained general acceptance (4).

3.



Surface-active (detergent) substances

4.



These substances are equally effective against gram-positive and gram-negative bacteria. They vary somewhat in activity. Zephiran, for example, is bactericidal to staphylococcus in dilutions as high as about 1:40,000 and bacteriostatic in dilutions as high as 1:200,000. The phenol coefficient of this compound has been found to range from 150 to 300 at room temperature against both staphylococcus and the typhoid bacillus, but the applicability of the phenol coefficient method to the assay of the activity of compounds of this kind is open to question.

There is a marked incompatibility between anionic and cationic detergents in that in mixture the antibacterial activity disappears. The non-ionic detergents do not have this effect and, for example, quaternary ammonium compounds may be mixed with non-ionic detergents having good solubilizing activity to give an antibacterial cleansing agent.

Alcohol and ethers. Ethyl alcohol and ethyl ether, often used as skin disinfectants,[63] are not very good germicides. Their effectiveness probably lies in the solution of the lipoidal secretions of the skin and consequent mechanical removal of microorganisms. Absolute alcohol has little or no germicidal activity. The bactericidal activity of alcohol-water solutions increases with the addition of water, but 50 per cent alcohol and less has little activity; 70 per cent is the concentration usually used for skin disinfection. Absolute propyl and isopropyl alcohols are likewise ineffective but show activity in aqueous solution, while absolute methyl alcohol is said to be bactericidal.

Gaseous Disinfectants.[6] The use of bactericidal gases for the disinfection of rooms, dwellings, and the like (fumigation or terminal disinfection) has declined markedly in recent years with no coincident increase in the prevalence of infectious disease. The commonly used gas, sulfur dioxide (generated by burning flowers of sulfur), is probably not bactericidal as a gas but is bactericidal in aqueous solution; it is effective, therefore, only in the presence of adequate amounts of moisture (a relative humidity of 60 per cent or higher). Other gases such as hydrogen cyanide have little or no effect on bacteria. Although the value of terminal disinfection is open to serious question, that of disinfestation is well established and the gases, hydrocyanic acid in particular, are widely used for the destruction of rats aboard ship, and like purposes.

Alkylating agents. Certain of these have been found to be highly effective bactericides in gaseous form, both on solid objects and solutions, and on bacteria suspended in air. These include ethylene oxide, propylene oxide, ethylene amine, methyl bromide, and formaldehyde. These substances have the unusual property of being relatively more effective in the destruction of bacterial spores than the usual disinfectants and their activity seems to be irreversible and bactericidal. Ethylene oxide has been investigated the most thoroughly. It forms an explosive mixture with air, but the attendant danger is prevented by mixing with 7 to 10 volumes of carbon dioxide; the mixture is commonly called *carboxide*. It may be applied to fabrics or equipment of various kinds under pressure; the objects to be sterilized are treated in the dressing sterilizer type of autoclave which is evacuated to 14 pounds, and ethylene oxide run in to 20 to 23 pounds pressure and left overnight.[21]

It is of some interest too that culture mediums can be so sterilized, with apparently no residual antibacterial activity remaining. This method of sterilization is particularly applicable to plastic disposable petri dishes and test tubes which cannot be sterilized by the conventional heating methods.[8]

Another alkylating agent which has been of particular interest is β-propiolactone.[7, 10, 32, 95] Unlike ethylene oxide, it is not inflammable, but requires a high humidity (80 per cent) to be effective, and has only limited penetrating power so that it functions most effectively as a surface disinfectant. It is highly bactericidal and virucidal in concentrations of 1–5 mg. l. and is considered to be about

25 times more effective than formaldehyde. While lacking the general utility of ethylene oxide, it is especially effective in the decontamination of enclosed spaces such as rooms and buildings. It is viewed with reservation by some because it has been found to be carcinogenic in the mouse.

Aerosols. An aerosol may be defined as a finely dispersed substance in air, and aerosols are of interest in connection with the destruction of air-borne microorganisms. Suitable substances must not only have antimicrobial activity, but also be sufficiently nontoxic and nonirritating that they may be inhaled by man and other animals for reasonable periods. The most commonly used compounds are glycols, especially triethylene glycol.

The glycol is relatively nontoxic and the vapor may be breathed with impunity, but bactericidal concentrations of 50 per cent or higher are attained in contact with suspended microorganisms. The rate of destruction of air-borne bacteria is in agreement with the assumption that the glycol molecules in the vapor state condense on the bacteria-containing droplets, and efficacy is associated with a low vapor pressure, high hygroscopicity, and, of course, toxicity for the bacterial cell substance.[57, 78, 79]

While pathogenic microorganisms such as pneumococcus, streptococcus, and influenza virus are killed or inactivated rapidly under experimental conditions, in general aerosols have given disappointing results when used for practical control of air-borne infection.

Dyes. The dyes are widely used in bacteriology both for staining purposes and as indicators. In addition, many of them show a marked bacteriostatic and bactericidal activity which is often specific in that it is manifested against one organism and not another. The incorporation of an appropriate dye in a medium will render it selective, *i.e.*, it will favor the growth of some species of bacteria and inhibit that of others. In general, this specificity is correlated with the Gram reaction; the gram-negative organisms are, for the most part, much less sensitive to dyes than are the gram-positive species. The activity of these compounds is affected by pH, the toxicity of the acid dyes increasing with acidity and that of the basic dyes increasing with alkalinity.

Many of the dyes such as the thiazins, oxazins, and azo dyes are not particularly toxic for bacteria, dilutions of 1:1000 or less being required to inhibit growth. A number of the triphenyl methane dyes are, on the other hand, inhibitory in high dilutions. Malachite green, for example, inhibits the growth of *B. subtilis* in dilutions of 1:4,000,000 and staphylococci in 1:1,000,000, while higher concentrations, *i.e.*, 1:30,000 to 1:40,000, are required to inhibit the colon and typhoid bacilli. Victoria green, a dichlor derivative of malachite green, is bacteriostatic to about the same degree. Brilliant green is active in even higher dilutions, inhibiting *B. subtilis* in a dilution of 1:15,000,000 and staphylococci in 1:4,000,000, and the typhoid and colon bacilli in 1:500,000.

The bacteriostatic properties of the triamino-triphenyl methane dyes, the so-called rosanilins, are apparently associated with the substitution of alkyl groups in the amido side chains. Basic fuchsin, a mixture of the unsubstituted simple dyes rosanilin and pararosanilin, is relatively weakly bacteriostatic, dilutions of 1:500,000 being required to inhibit the growth of *B. subtilis.* Acid fuchsin, a mixture of various sulfonated derivatives of basic fuchsin, is likewise only weakly inhibitory and was formerly widely used in mediums as an acid indicator under the name of Andrade's indicator. On the other hand, methyl violet* a mixture of tetra-, penta- and hexamethyl pararosanilin, is markedly bacteriostatic and completely inhibits the growth of bacteria such as staphylococci, diphtheria bacilli, and others in dilutions of 1:1,000,000 to 1:5,000,000. Approximately 150 times as much dye is necessary to suppress growth of the less sensitive gram-negative bacteria such as

* Gentian violet is a more or less impure mixture of methyl violet and dextrin. Crystal violet, hexamethyl-*p*-rosanilin, is one of the constituents of methyl violet.

the colon and typhoid bacilli. There appears to be a correlation of bactericidal activity and basicity of these compounds.

The acridine dyes, acriflavine and trypaflavine, have been of particular interest because of their therapeutic significance. The former is actively bacteriostatic in dilutions as high as 1:3,000,000, and the latter inhibitive to staphylococci and similar organisms in 1:2,000,000 and to the relatively fragile gonococcus in dilutions of 1:10,000,000 to 1:50,000,000. The gonococcus is killed by exposure to the dye in dilutions of 1:80,000 to 1:400,000 within two or three minutes.

The Mechanisms of Bactericidal Action. The ways in which antimicrobial substances affect the cell so as to produce changes in its organization resulting in growth inhibition or death are varied and, as might be expected, dissimilar effects on the cell give the same end result. A substance showing bactericidal properties produces its effects through an interruption of essential physiological processes of the bacterial cell. In some instances the effect is relatively nonspecific, as in the denaturation of constituent protein, and in others highly specific in that some particular metabolic reaction is affected. There is some tendency to differentiate the former as a general protoplasmic poisoning, but this is more a matter of convenience than an expression of fundamental difference. A continuous series of degrees of specificity, so to speak, may be set up through which the general toxic effects grade imperceptibly into the inhibition of a single enzyme system.

Thus, a general oxidation of cell substance by oxidizing agents, a saturation of thiol groups of constituent proteins with heavy metals, or a generalized denaturation of protein by, for example, substitution of halogens such as iodine in the ring structures of aromatic amino acids, obviously alters the organization of the cell on a macromolecular level to such an extent that normal metabolism cannot continue. Bactericidal substances of the phenol group apparently act in part in this way in that phenol is surface-active and by orientation of the hydroxy group reacts with free amino groups of the cell proteins.

Or the effect may be somewhat more localized as in the case of the basic dyes which react with nucleoprotein and whose action, at least that of acriflavine, is antagonized by nucleotides. Similarly, the surface-active detergents are antagonized by lipids, *e.g.*, the anionic detergents by cephalin, and there is some reason to believe that they alter the permeability of the cell wall to such an extent that the cell dies. Phenols and other substances act similarly, and in general the antimicrobial effect of such agents is a net one, with leakage of cell contents due to permeability effects, possibly general protein denaturation, and adverse effects on the availability of coenzymes, activating ions, etc.

Generally speaking, the loss of enzyme activity is the most important single factor,[95] and even antimicrobial substances that appear to be general protoplasmic poisons often show a specificity of action. There is evidence that chlorine, for example, first affects a triosephosphate dehydrogenase system. Sulfhydryl-containing enzymes appear to be the most susceptible to the action of bactericidal substances, and poisoning by heavy metals, oxidizing agents, etc., occurs with some facility. The alkylating agents such as ethylene oxide act by direct alkylation, and the thiol group is probably the most vulnerable point in the cell organization. Azide markedly inhibits esterification of inorganic phosphate, and it has been suggested that it reacts with the acyl phosphate of diphosphoglycerate. The acyl azide is spontaneously decomposed and thus drains the energy-rich phosphate formed.

Often antimicrobial activity is paralleled by an inhibition of oxygen uptake, indicating a poisoning of the respiratory enzymes, and measurement of such inhibition has been suggested as a method of assaying bactericidal activity. This approach is not always valid, however, for some substances such as dinitrophenol stimulate oxygen uptake and prevent the assimilation of carbon; it inhibits assimi-

lation by uncoupling phosphorylation and draining away the energy stored in energy-rich phosphate bonds. It will be clear that while information as to the precise point of attack of antimicrobial substances on cell organization and function is by no means complete, a variety of mechanisms are operative.

Reversal of antibacterial activity. Microorganisms exposed to the action of a number of antimicrobial substances and apparently killed may be "revived" by neutralization of the activity within a short time after exposure. The revival of bacteria and viruses such as influenza virus, presumably dead after treatment with mercuric chloride, by treatment with hydrogen sulfide or thiols such as thioglycollic acid, glutathione, and the like is well established. Similarly, bacteria apparently killed with hypochlorite may be revived by inactivating the chlorine with sodium thiosulfate, and the use of thiosulfate solutions for dilution in titration of bactericidal activity of the hypochlorites to neutralize bacteriostatic concentrations carried over into subcultures is a common procedure. Bacteria treated with phenol survive if the phenol is removed by adsorption on charcoal or treatment with ferric chloride. The effects of treatment with dyes such as acriflavine or with cationic detergents may be neutralized with survival of the bacteria by high molecular weight anions or, in the case of dyes, more simply by shifting the pH so that the dye-protoplasm complex is no longer un-ionized, provided that the shift is within physiological limits.

In the case of bactericidal concentrations of the compound, it is not clear how far the bactericidal action can be allowed to go and yet permit revival. In many instances it is probable that it has not gone far, perhaps little beyond a preliminary sorption. When the compound is in bacteriostatic concentration only, of course, the cells remain alive for relatively long periods and multiply as soon as the inhibition is removed by neutralization or simple dilution. The significance of reversal of the antimicrobial effect lies in the inferences that may be drawn as to the nature of the antimicrobial activity.

The specificity of disinfectants. The marked specificity of the bacteriostatic and bactericidal activity of the dyes has been referred to above. The property of differential toxicity is not confined to these compounds alone, however, but is exhibited to some degree by many of the bactericidal chemicals. The hypochlorites, for example, while powerful germicides for most bacteria, have little effect on the tubercle bacillus, and it is of practical significance also that poliovirus persists in treated waters containing sufficient chlorine to destroy pathogenic bacterial contaminants.[55] In general, the salts of the heavy metals are the least specific in their action and dyes the most, with other compounds lying between these two extremes. Certain slow oxidizing agents such as potassium dichromate exert a selective bacteriostatic effect on gram-negative bacteria, and iodine is more efficacious against these microorganisms. Similarly, among the long-chain aliphatic bases, the less strongly basic amines act on gram-positive bacteria for the most part, whereas the gram-negative organisms are more susceptible to the action of the stronger bases such as guanidines and quaternary amines. In general, the viruses are considerably more resistant to the activity of quaternary ammonium compounds than are bacteria. These compounds fail, for example, to inactivate vaccinia virus in dilutions as low as 1:1000 although they are bactericidal in many thousandfold dilution.

Factors influencing disinfection. The process of disinfection or bacterial death is often, in part at least, a chemical reaction and is, therefore, subject to a variety of influences which affect the velocity of such reactions. The most important of these influences is the concentration of the reacting substances, *i.e.*, the concentration of disinfectant and the numbers of bacteria present. The *effective* concentration of disinfectant is, in turn, dependent upon two other factors:

first, the presence of moisture, which makes possible coagulation by heat and ionization of the bactericidal salts, and acts as a solvent and suspending medium in which there may be intimate contact between the disinfectant and the microorganism; second, the presence of extraneous organic matter.

Many chemical disinfectants act through a combination with the protein of the cell and, if extraneous organic matter is present, will, of course, react with this inert material, thereby reducing the effective concentration. Disinfectants vary widely in the degree to which their bactericidal properties are affected by organic matter. The salts of the heavy metals are rapidly precipitated by organic material, while compounds such as phenol and the cresols are only slightly affected. The rate of destruction by heat is also affected by the presence of organic matter—organisms embedded in a mass of fecal material, for example, are protected from heat for a short time. The process of disinfection by germicides or by heat is influenced by temperature, the velocity of the reaction increasing with rise in temperature.

The pH likewise influences the rate of bacterial destruction not only by heat but by many chemical compounds, the velocity, in general, being least at neutrality and increasing with increase in acidity or alkalinity. A number of other factors, such as the presence of salts,[117] affect the rate of disinfection but generally not sufficiently to be of practical importance.

From the practical point of view, the time of exposure of bacteria to a given disinfectant is of considerable significance and bears an inverse relation to the rapidity of killing. The time allowed for the destruction of bacteria is determined not only by the factors discussed above but also by the kind of bacteria that are to be killed. In certain cases the specificity of a disinfectant may be so marked that it must be taken into consideration. For example, the relative atoxicity of hypochlorite for the tubercle bacillus referred to above precludes its use in the disinfection of tuberculous sputum. Bacterial spores are much more resistant to heat and chemical disinfectants than are the vegetative cells, and considerably more time must be allowed for their destruction. The vegetative cells of some bacteria may be somewhat more resistant than those of others, but, for the most part, such differences are too small to be of practical significance.

The dynamics of disinfection.[103] Quantitative studies on the rate at which microorganisms are killed by lethal agents have indicated that in

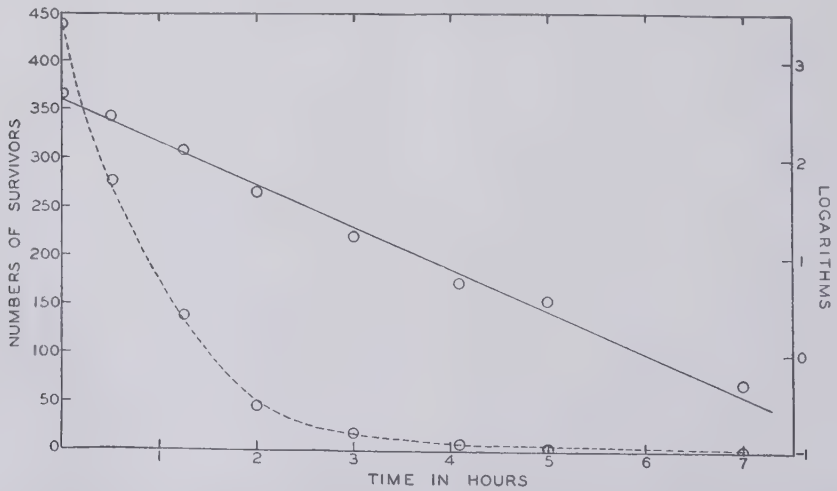


Figure 48. Death rate of anthrax spores treated with 5 per cent phenol. The dotted line is the arithmetic plot and the solid line the logarithmic plot. The negative logarithm was obtained by taking samples of several times the unit volume. (After Chick.)

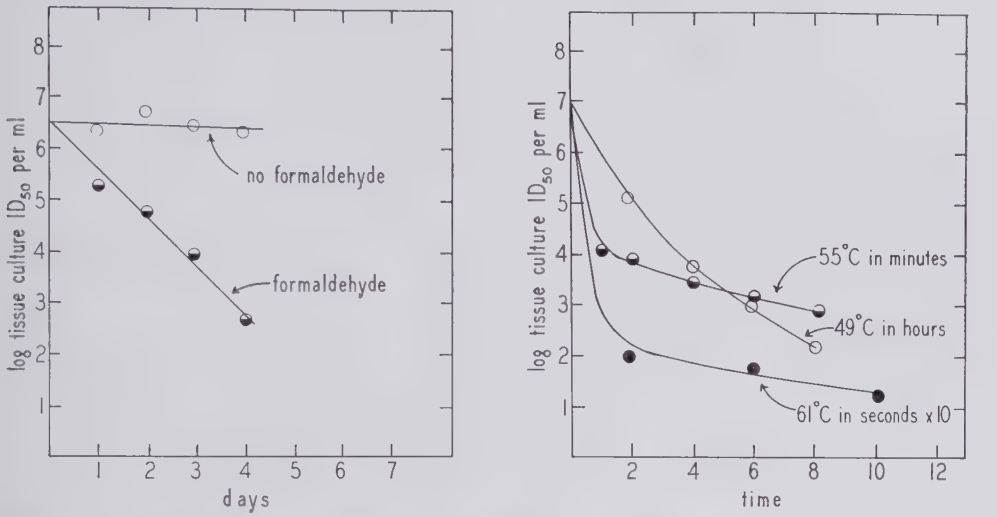


Figure 49. The inactivation of tissue culture foot-and-mouth disease virus by 0.009 per cent formaldehyde (*left*) and by heat (*right*). The formaldehyde inactivation is exponential over the indicated range, but deviation from an exponential rate during heat inactivation is marked, with increased heat stability at low survival levels. (Data from Bachrach, Breese, Callis, Hess, and Patty.)

many instances the organisms die at a logarithmic rate, *i.e.*, if the logarithms of the numbers of viable organisms are plotted against time, the points tend to fall on a straight line. This phenomenon has been observed in the death of both spores and vegetative cells under the influence of chemical disinfectants or moist heat and also occurs in the death of bacteria in old cultures. The velocity of the reaction, the slope of such a line, depends upon the concentration and kind of disinfectant, the nature of the organisms—whether spores or vegetative cells—and other factors which influence the process of disinfection. This logarithmic rate likewise describes the course of a monomolecular reaction, and this fact has led some to conclude that bacterial death is a monomolecular chemical reaction. Although the killing of bacteria by some disinfectants is undoubtedly a chemical process, as for example the precipitation of constituent protein as proteinates of heavy metals, the evidence does not justify the conclusion that the reaction is monomolecular. The fallacy is the old one of confusion of correlation and causality under the slightly different guise of description and mechanism. Experimental evidence has indicated that while such semilogarithmic plots may often be fitted with a straight line, others are best fitted by sigmoid curves, the death rate being more rapid in the beginning in some cases and more rapid at the end in others or even highly irregular.

One of the more important determining factors is a graded biological resistance of the cells in the bacterial population, an explanation that is supported by general biological considerations. Undoubtedly the phenomenon of bacterial death is a complex of inter-related factors whose admixture with the mechanics of the mass law results in the parameters determined by mathematical means. Of practical importance, however, is the fact that in disinfection by chemicals and by heat there is a minority of cells, possibly more resistant, that survive long after the majority have perished and that must be destroyed in order to obtain complete sterilization. The determination of antiviral activity poses special technical problems because of the necessity for assay of viable agent by infectivity titrations,[44] and the effects of auto-interference when a large portion of the agent has been inactivated toward the end point (Chap. 4).

In any case, it is clearly not possible to extrapolate the exponential death rate

to zero and assume that the time of exposure so indicated insures sterility; such an assumption resulted, for example, in incomplete inactivation of poliovirus by formalin in the preparation of vaccines.[114]

The Standardization of Disinfectants. The relative bactericidal efficiency of the chemical disinfectants is a point of considerable practical importance. The value of a quantitative method for the determination of the killing power of germicides was recognized early in the development of bacteriology, and experimental investigation led to the establishing of a standardized technique which made possible the determination of the bactericidal power of a given chemical compound relative to that of phenol. The numerical value so determined is termed the *phenol coefficient* and is presumed to indicate whether, and to approximately what extent, the unknown is a better or poorer germicide than phenol. Later methods of standardization have grown out of this procedure. The phenol coefficient of a disinfectant used against the typhoid bacillus is calculated as follows:

Divide the greatest dilution of the disinfectant capable of killing *Salmonella typhi* in 10 minutes but not in 5 minutes by the phenol dilution which so kills and divide these figures one into another. In order not to convey a false idea of the accuracy of the method the coefficient is calculated to the nearest 0.1 point if under 1, to the nearest 0.2 point if between 1 and 5, to the nearest 0.5 point if between 5 and 10, and to the nearest 1.0 point if between 10 and 20. The conditions regarded as standardized in the United States, the FDA phenol coefficient, have been defined by the Food and Drug Administration.

The effect of extraneous organic matter on the bactericidal power of a disinfectant is commonly taken into consideration by carrying out the test with and without added organic matter. Three per cent of dried fecal matter or dried yeast may be added to the bacterial suspension or the organisms may be suspended in 50 per cent serum. It is important to recognize that bacteria differ considerably in their resistance to phenol, staphylococci, for example, being much more resistant than the typhoid bacillus, so that in strict accuracy it is necessary to specify "typhoid phenol coefficient," "pneumococcus phenol coefficient," etc.

Determination of the Phenol Coefficient*

DISINFECTANT	DILUTION	TIME IN MINUTES		
		5	10	15
unknown	1:300	0	0	0
	1:325	+	0	0
	1:350	+	0	0
	1:375	+	+	0
	1:400	+	+	+
phenol	1:90	+	0	0
	1:100	+	+	+

The test is satisfactory only when the resistance of the test organism, here *Sal. typhi*, gives one or another of the following reactions:

1:90	+ or 0	+ or 0	0
1:100	+	+	+ or 0

$$\text{phenol coefficient} = \frac{350}{90} = 3.89 = 3.9$$

* From Stuart, in Reddish, G. F.: Antiseptics, Disinfectants, Fungicides, and Chemical and Physical Sterilization. 2nd ed. Philadelphia, Lea and Febiger, 1957.

The phenol coefficient criterion of antimicrobial activity has many defects, including failure to evaluate concentration effects, temperature coefficients, etc. In addition, the validity of a phenol coefficient determined for a nonphenolic disinfectant is open to serious question. In consequence, many variations of the procedure have been used. For example, the *germicidal equivalent concentration test* has been developed for the evaluation of the lethal activity of substances such as hypochlorites, in which concentrations giving the same killing pattern as those of a reference compound are determined. This kind of test is illustrated in the accompanying tabled example; here the activity of the unknown disinfectant in a concentration of 10 ppm is equivalent to that of the reference standard, NaOCl, in a concentration of 50 ppm.

Assay of the antimicrobial activity[112] of detergents is difficult because of the tendency of the test bacteria to become adsorbed to the walls of the test tube as

The Germicidal Equivalent Concentration Test*

DISINFECTANT	CONCENTRATION (in ppm)	SUBCULTURE SERIES**									
		1	2	3	4	5	6	7	8	9	10
unknown	10	0	0	+	+	+	+	+	+	+	+
	20	0	0	0	0	+	+	+	+	+	+
	25	0	0	0	0	0	+	+	+	+	+
NaOCl control	50	0	0	+	+	+	+	+	+	+	+
	100	0	0	0	+	+	+	+	+	+	+
	200	0	0	0	0	0	0	+	+	+	+

* From Stuart, in Reddish, G. F.: Antiseptics, Disinfectants, Fungicides, and Chemical and Physical Sterilization. 2nd ed. Philadelphia, Lea and Febiger, 1957.

** The time interval may vary from 30 seconds to 2 minutes.

a consequence of altered charge in the presence of the disinfectant. So-called *use dilution tests* have been employed for these substances in which a suspension of the test microorganism is dried on the surface of a carrier such as glass rods or rings, immersed in the disinfectant for varying periods, and subsequently cultured. Such methods are not new but were used many years ago by Koch and others who dried anthrax spores on silk threads, glass beads, etc., for testing bactericidal activity.

All of these methods of assay suffer from the fundamental defect of dependence upon a sterility end point, an end point which studies of the dynamics of the process of disinfection have shown to be highly questionable.

It is generally agreed that the rate of killing, *i.e.*, the reaction velocity constant, is a much more accurate measure than any value whose derivation depends upon a sterility end point. The relation between concentration of disinfectant and time required for killing is an exponential one, and the reaction velocity constant, k, is given by the expression:

$$k = C^n t$$

when C is the concentration, n a constant characteristic for each disinfectant, and t is time. The temperature coefficient may, of course, be determined experimentally for each disinfectant. It will be clear that this kind of characterization of the bactericidal activity of a given compound is much more informative than the usual type of phenol coefficient, no matter how precisely the conditions of the test are defined. It is not, however, generally used for routine work.[43, 48, 49, 50, 51, 52, 53, 54, 119, 120]

The practical value of a disinfectant is not always indicated by tests made under the controlled conditions of the laboratory. Hydrogen peroxide, for example, may give a quite respectable phenol coefficient but, when applied to an abrasion, is so rapidly decomposed under the influence of tissue catalase that its germicidal powers are almost immediately exhausted. This has led to various kinds of *infection prevention tests* which have necessarily included the matter of the toxicity of the disinfectant for tissue cells.

A given disinfectant may be so highly toxic for tissue cells that it has no practical value. Salle and his associates[87] have proposed a "toxicity index" which takes into consideration not only the germicidal activity of a compound but its toxicity for tissue as well. For example, Witlin[121] determined the concentration of bactericidal substances lethal for the chick embryo and calculated a toxicity index by using the concentration of the bactericide in grams per milliliter killing the test organism, *Staphylococcus aureus*, in 10 minutes but not in 5 as the numerator, and the chick embryo MLD in grams for the denominator. Representative values were: phenol (1:20)—1.18; $HgCl_2$ (1:1000)—0.62; tincture of iodine –0.044; sodium hypochlorite—1.39; Mercurochrome (1:50)—13.3; and values

ranging as high as 9.1 for various organic mercurials. Spaulding and Bondi[92] have developed an infection-prevention-toxicity test in which the tip of a mouse tail is contaminated with bacteria, the tail dipped into the solution of disinfectant, and then the tip is removed and placed in the peritoneal cavity. The highest dilution of the disinfectant protecting 50 per cent of the mice is taken as the numerator, and the greatest concentration allowing survival (from poisoning) of the test animal is taken as the denominator, to give a ratio or infection-prevention–toxicity index. Such toxicity indexes are of considerable practical importance in the case of skin disinfectants.

The whole question of the evaluation of disinfectants is beset with many difficulties, both theoretical and practical, and to date remains an open one to which no entirely satisfactory answer has been supplied.

THE CHEMOTHERAPEUTIC DRUGS[20, 108, 109, 126]

Antibacterial activity as described previously is characterized by its manifestation *in vitro, i.e.,* against the living microorganism but outside the body of the host in the case of the pathogenic microorganisms, and by the end point of bacterial death, a criterion of bactericidal activity. The feasibility of using antibacterial substances *in vivo* as chemotherapeutic agents is dependent primarily upon the phenomenon of specificity of action of such substances, specificity as regards selective action on the microorganism without significant harmful effects on the host. This idea is by no means new; it was the basis of Ehrlich's search for a "magic bullet," a compound strongly germicidal for a given microorganism yet sufficiently nontoxic that it could be injected in suitable amount to give effective concentrations in the tissues. His work, originally directed toward the therapy of African sleeping sickness of trypanosome etiology, was an attempt to retain the antimicrobial activity of arsenic compounds and at the same time reduce toxicity for the host, and culminated in the synthesis of salvarsan. The original work has, of course, been greatly extended and amplified both by Ehrlich and other workers through the preparation of other arsenicals, antimony compounds, and so forth.

Prior to 1940 the approach to chemotherapy of infectious diseases was fundamentally the same, a trial-and-error search for antimicrobial activity *in vivo,* followed by determination of the active portion of the molecule, and retention of this activity with coincident reduction of toxicity to the host by the synthesis and testing of a great number of related compounds. For example, the small but significant antimalarial activity of the substituted thionine, methylene blue, was exploited by the German workers in the development of Atabrine. A general practice, then, has been the routine testing of synthetic organic chemicals, especially dyestuffs and dye intermediates, for antimicrobial activity *in vivo.* This procedure led Domagk[13] in 1935 to the observation of the marked chemotherapeutic activity of the dye Prontosil in experimental streptococcus infections. Routine procedure led to identification of the active portion of the molecule, *p*-aminobenzene sulfonamide (sulfanilamide), and the synthesis of a great number of related compounds known as the sulfonamides. While of great practical significance in that these compounds were the first found to be effective in bacterial infections, the discovery and study of the sulfonamides marks a great step forward in that (1) there is now available for the first time a rational theory of chemotherapy (see below), and (2) it is now clear that effective chemotherapeutic agents are usually bacteriostatic rather than bactericidal.

As implied earlier, the differentiation

of bactericidal and bacteriostatic activity is essentially one of convenience based on the concentration differential of the active substance, *i.e.*, when the difference between the two end points (growth inhibition and death) is great, the compound is regarded as primarily bacteriostatic, but when it is very small the bacteriostatic zone is so limited as to appear unimportant and the term bactericidal is usually applied. Compounds that are practical chemotherapeutic agents are, like the triphenylmethane dyes, bacteriostatic substances.

The chemotherapeutic activity of a substance employed in bacteriostatic concentrations rests in large part upon the host facet of the host-parasite relationship. Upon invasion of a susceptible host with a pathogenic microorganism, a series of mechanisms grouped under the head of resistance or natural immunity (Chap. 9) become functional. The relationship between the host and the microorganism, then, is a dynamic one which is affected by lesser factors influencing one or the other. Antitoxin therapy, for example, reinforces the immune response of the host, tipping the balance against the microorganism. Similarly, the administration of a substance that does not interfere to a significant extent with the mechanisms of natural immunity, but does prevent the microorganism from proliferating within the tissues, also tips the balance against the microorganism to allow its destruction and elimination by the host. Formerly the role of host defense was not fully appreciated, and the significance of the failure to take this factor into account is underscored by the fact that sulfonamide compounds were investigated by one of the outstanding research laboratories as early as 1912 and discarded because they showed little bactericidal activity.

At the present time a number of highly effective chemotherapeutic agents are known. They may be divided into two groups: those that were originally synthetic organic chemicals; and those that are derived from living organisms. The latter may be divided again into two subgroups, those substances that are formed by certain species of bacteria, and those that are formed by actinomycetes and molds. The structures of some of the naturally occurring substances are known, and one is prepared on a commercial scale by synthesis. All are characterized by being primarily bacteriostatic and by a specificity of action manifested not only by a lack of toxicity for the host, but also by differential activity with respect to species, and sometimes strains, of microorganisms.

SYNTHETIC COMPOUNDS

The synthetic chemotherapeutic agents, with the exception of the sulfonamides, are used in the therapy of diseases produced by the animal parasites and in the treatment of tuberculosis and leprosy. The first group is considered elsewhere (Chap. 34). The chemotherapeutic agents having greater or lesser efficiency in the treatment of infections with the acid-fast bacilli fall into four groups, *viz.*, the sulfones, the aminohydroxybenzoic acids, the thiosemicarbazones, and the derivatives of pyridine carboxylic acid.

The Sulfones. The antituberculous activity of the parent compound of the sulfone group, 4,4′-diaminodiphenylsulfone (DDS, DADPS, Dapsone), was reported by French workers in 1940. Various disubstitution products, Promin (sodium 4, 4-diaminodiphenyl sulfone-N, N′ didextrose sulfonate), Promacetin (sodium 4,4′-diaminodiphenylsulfone-2-acetylsulfonamide), Diasone (disodium formaldehyde sulfoxalate diaminodiphenyl sulfone), and Sulphetrone (4,4′-bis [γ-phenyl-N-propylamino] diphenylsulfone-tetrasodium sulfonate), have been prepared in an effort to reduce toxicity and increase solubility and activity. None has been particularly successful, possibly in part because it is probable that the active derivatives are hydrolyzed to DDS *in vivo* and the activity is attributable to that of the parent compound. In general, the activity and toxicity of the sulfones are such that they are not highly effec-

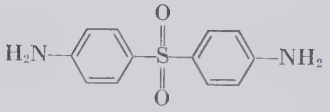
tive chemotherapeutic agents and are not generally used in the treatment of tuberculosis since better drugs are available. However, these compounds are useful in the treatment of leprosy.

Aminohydroxybenzoic Acids. Of these compounds, *p*-aminosalicylic acid (PAS) is the best known. Its activity against the tubercle bacillus was reported in 1944, based on the observation made three years earlier that the oxygen uptake of the tubercle bacillus is increased in the presence of salicylates and benzoates. It is relatively nontoxic and is currently most useful when administered together with streptomycin, since it markedly reduces the tendency of tubercle bacilli to become resistant to the antibiotic (Chap. 31).

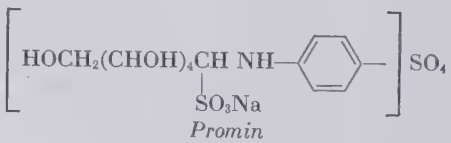
Many derivatives have, of course, been prepared, but of these only one, phenyl *p*-aminosalicylate (FR 7), first described in 1951, shows greater activity than PAS—about tenfold when given subcutaneously.

Thiosemicarbazones. These compounds, described as possible chemotherapeutic agents in 1946, are more active against the tubercle bacillus than are the sulfones and PAS. The parent compound (TB I) is not particularly active, but *p*-acetaminobenzaldehyde thiosemicarbazone (Tibione, TB 1/698, Conteben, Thiacetazone, Amithiozone) has considerable activity and has been widely used in Europe, where it has been found reasonably effective in most forms of tuberculosis. It is, however, toxic (gastrointestinal disturbances, anemia, liver, and kidney damage), and the incidence of severe side reactions is high. Many derivatives of this structure have been prepared, most of them involving alteration in the character and position of the subordinate grouping on the benzene ring. Of these, only the *p*-ethylsulfonyl compound (TB III) appears promising. Of the three isomers of the pyridine analogue of Tibione, the β and γ isomers have shown promise in experimental tuberculosis, but have not been widely tested.

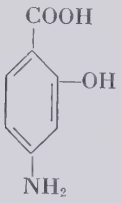
5.



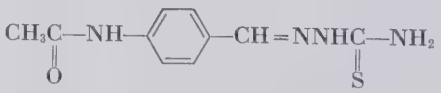
4,4′ diaminodiphenyl sulfone



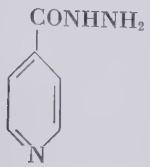
Promin



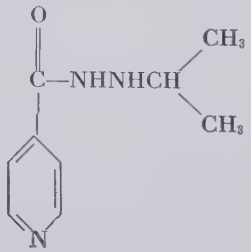
PAS



Tibione



isonicotinic acid hydrazide



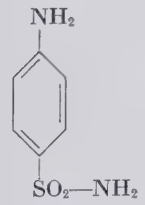
1-isonicotinyl-2-isopropylhydrazine

Pyridine Carboxylic Acid Compounds. The tuberculostatic activity of nicotinamide was reported in 1945; the activity is not, as stated by some workers, a function of its vitamin activity since nicotinic acid has no antibacterial activity. Isonicotinic acid hydrazide (Rimifon, Nydrazid, Neoteben, INH) and 1-isonicotinyl-2-isopropylhydrazine (Marsilid) have been found to have marked activity, several-fold greater than streptomycin in experimental tuberculosis. Further study has shown that activity is not necessarily limited to immediate derivatives of nicotinamide, but may be associated with the pyridine structure, and pyrizinamide (Aldinamide), for example, has chemotherapeutic activity.

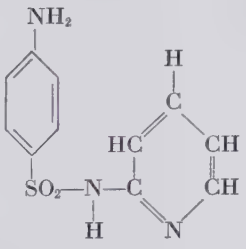
Sulfonamide Compounds.[28, 107] As indicated above, *p*-aminobenzene sulfonamide was the first of the chemotherapeutic substances effective in bacterial infections other than the treponematoses that are susceptible to treatment with arsenicals. The term sulfonamide is generally taken to include the parent compound and its derivatives. Several thousand sulfonamides have been prepared, usually by substitution in the amido group attached to the sulfone radical, but some, such as Sulfathalidine and Sulfasuxidine, contain substitutions in the amino group attached directly to the benzene ring. These last are poorly absorbed from the intestinal tract and have considerable utility in some infections confined to the lumen of the bowel, such as bacillary dysentery. Of all the compounds prepared, only a few have been generally used. They are effective against microorganisms such as the streptococci, staphylococci, pneumococci, gonococcus, meningococcus, the plague bacillus, the dysentery bacilli, but are relatively ineffective against microorganisms of the Salmonella group including the typhoid bacillus, rickettsiae, and others.

There appears to be little qualitative difference among these compounds so far as antibacterial activity is concerned, but they differ with respect to solubility, rates of absorption and excretion, and other factors. In general, they are only sparingly soluble in water, and solubility increases with alkalinity. On absorption a portion of the drug is inactivated by combination with plasma protein, and a portion acetylated to an inactive form in the liver, and both active and inactive drug are excreted in the urine. When

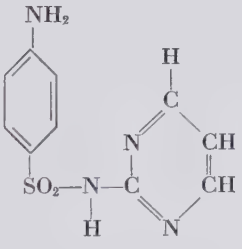
6.



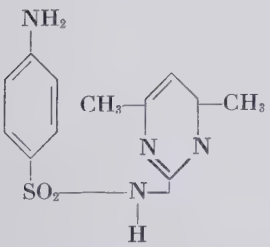
sulfanilamide



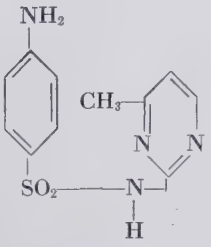
sulfapyridine



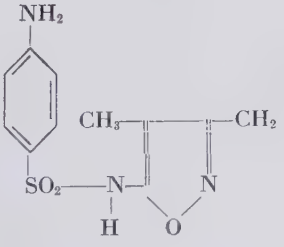
sulfadiazine



sulfamethazine



sulfamerazine



Gantrisin, gantrosan, sulfisoxazole, sulfafurazole

the urine is acid and its volume low, the drug may accumulate in the kidney with resulting damage. The administration of mixtures of sulfonamides, such as triple sulfa containing sulfadiazine, sulfamethazine and sulfamerazine, has no direct therapeutic advantage, but reduces precipitation in the kidney since the solubility of each is independent of the presence of the others.

At the present time the sulfonamides are somewhat neglected as a consequence of the glamor of the antibiotics. While in many, but not all, instances they are less potent therapeutic agents, the sulfonamides will do just as much, more simply and cheaply, though possibly more slowly, than the antibiotics. They have the additional advantage that their administration produces less drastic changes in the normal flora and consequently less secondary infection with drug-resistant microorganisms.[129]

THE ANTIMETABOLITE THEORY OF ANTIBACTERIAL ACTIVITY[80, 88, 90, 123, 124, 125]

Competitive inhibition of enzyme reactions in which a structural analogue of the substrate competes with the substrate for the enzyme was described by Quastel and Wooldridge in 1928. They demonstrated the inhibition by malonic acid of the oxidation of succinic acid to fumaric acid catalyzed by succinic dehydrogenase in the presence of a hydrogen acceptor, and reversal of the inhibiting effect by excess succinate. Malonic acid differs from the substrate in that it is a 3- instead of a 4-carbon dicarboxylic acid.

If such a reaction is an essential part of the metabolic processes of a bacterium, the inhibitor exhibits antibacterial activity. The immense practical significance of this was pointed up in the observation by Woods in 1940 that the antibacterial activity of sulfonamide is specifically inhibited by *p*-aminobenzoic acid, a compound that is required as a bacterial vitamin by a number of organisms, including *Clostridium acetobutylicum, Acetobacter suboxydans,* and some strains of the diphtheria bacillus. The close similarity in the structure of *p*-aminobenzoic acid and *p*-aminobenzene sulfonamides will be obvious from the structural formulas in 6. That *p*-aminobenzoic acid is required by some bacteria suggests that it is involved in some essential metabolic reaction, and it was suggested by Woods and by Fildes that the action of the sulfonamides on bacteria is the result of a competition of these compounds with an essential bacterial metabolite, *p*-aminobenzoic acid. This inhibitory effect of sulfonamides is reversible and can be antagonized by excess *p*-aminobenzoic acid. This has the practical value of allowing successful blood culture from individuals undergoing sulfonamide therapy by inclusion of *p*-aminobenzoic acid in the culture medium (5 mg. per 100 ml.).

The generalization follows that essential physiological reactions of the microorganisms may be inhibited by analogues of metabolites, and that such antimetabolites might be expected to show antibacterial activity. The plausibility of such a hypothesis is underscored by the essentially growth-inhibiting rather than lethal effects of chemotherapeutically effective substances on pathogenic microorganisms under physiologically tolerable conditions. This hypothesis assumes primary importance by providing, for the first time, a rational basis for the chemotherapy of infectious disease.

Enzyme Inhibition. This theory is substantiated by impressive evidence. It has also been extended by the production of symptoms of vitamin deficiency in higher animals receiving vitamin analogues (pyrithiamine, glucoascorbic acid, isoriboflavin, β-acetyl-pyridine, etc.) and by encouraging results in the treatment of certain kinds of cancer with folic acid antagonists and with purine antagonists such as 6-mercaptopurine, 2,6-diaminopurine, 6-chlorpurine, and pyrazolo purines. Enzyme inhibition is also the basis of the activity of a number of narcotics and of certain chemical warfare agents

and insecticides such as tetraalkyl phosphates, dialkyl *p*-nitrophenylphosphates, and dialkyl fluorophosphates, which irreversibly inhibit esterases, including acetylcholinesterase.

Generally, enzyme inhibition may be of a number of kinds, *viz.*, competitive inhibition of the kind typified by the malonate-succinate competition for succinic dehydrogenase noted above, noncompetitive inhibition in which the inhibitor combines with the enzyme at a site other than the active site, and, less commonly, other kinds of inhibition in which, for example, the inhibitor combines with the enzyme-substrate complex, removes essential ions by chelation, and so on. Competitive inhibition may be distinguished by plotting the degree of inhibition against substrate concentration; in the competitive type of inhibition this is a linear function of substrate concentration, *i.e.*, inhibition is inversely related to the ratio of substrate concentration to inhibitor concentration, while in the noncompetitive types in which the inhibitor does not combine with the enzyme at the active site, the affinity of the enzyme for substrate is not altered, and the rate of the enzyme-catalyzed reaction is not related to the ratio of the concentration of substrate to that of inhibitor. Such enzyme inhibitions may be reversible, or partially or completely irreversible.

The folic acid system. Inhibition among microorganisms may be illustrated by elaboration of the sulfonamide–*p*-aminobenzoic acid relationship. As described elsewhere (Chap. 5), *p*-aminobenzoic acid is an intermediate in the synthesis of folic acid, and folic acid is, in turn, a precursor of the formyl-folic acid (folinic acid) coenzyme. The synthesis of folic acid from *p*-aminobenzoic acid, glutamic acid, and the pteridine nucleus is antagonized by sulfonamides, and the inhibition is competitive. Sulfonamides do not, however, inhibit the conversion of folic acid to folinic acid; rather, this reaction is inhibited noncompetitively by 4-amino derivatives of folic acid, such as aminopterin and α-methopterin. The latter inhibition, then, prevents folic acid from acting as a formylating agent in 1-carbon transfer reactions involved in the synthesis of at least some amino acids and nucleic acids.

Differences in the effect of sulfonamides on different kinds of microorganisms may be resolved in terms of their synthetic abilities, and it has been suggested that three general groups may be distinguished. The first is the gram-negative bacteria which do not require preformed folic acid, and are inhibited by sulfonamides, and the inhibitory effect is not reversed by added folic acid. The second includes the gram-positive bacteria that do not require preformed folic acid, are inhibited by sulfonamides and the inhibition is reversed by folic acid. The third group, also gram-positive bacteria, is made up of those requiring preformed folic acid for growth and not sensitive to sulfonamides.

Limiting systems. In some instances analogues may compete with metabolites acting as coenzymes, but in general the evidence indicates that the competition occurs when the metabolite acts as a substrate rather than as a coenzyme. As a substrate, the metabolite is synthesized into a more complex molecule which functions as a coenzyme, or it may be degraded prior to synthesis. Further, various apo-enzymes may compete, for folic acid for example, and the effective ratio of inhibitor to metabolite depends upon which enzyme system is involved. The requirements of different systems vary, and one of them is the limiting factor.

The sulfonamide sensitivities of bacteria which do not require *p*-aminobenzoic acid or folic acid for growth may be abolished by including the amino acids, purines, and pyrimidines in the culture medium which are synthesized in the reactions catalyzed by folic acid derivatives. When the necessity for the functioning of the limiting system is removed by supplying its end product, the concentration of antimetabolite is no longer inhibitory. If, however, the antimetabo-

lite concentration is increased, inhibition is again apparent, another enzyme system has become the limiting factor, and again the inhibitory effect may be removed by supplying the end product of that system. Thus, the end products of the several systems involved which produce sparing effects can be arranged in an order. In the case of the colon bacillus, for example, methionine, purines, serine, and finally thymine or thymidine constitute such a series of which each member is dependent upon those preceding it. In this way studies of the nature of substances antagonizing the activity of the inhibitor may shed considerable light on the nature of the relevant reactions of synthesis.

Other inhibitors. There are a number of other instances of inhibition by analogues of essential metabolites of bacteria, produced by replacement of a carbonyl group with a sulfonic acid or sulfonamide radical or by a ketone, by substitution in the ring system of aromatic compounds, the replacement of alkyl side chains of ring systems with halogens, etc. Such are the sulfonic acid and sulfonamide analogues of nicotinic acid, pantolyltaurine (thiopanic acid), and pyrithiamine, the pyridine analogue of thiamine.

Inhibition may also be indirect or nonspecific in the sense that compounds other than structurally related analogues may be inhibitory. In the first instance the substance applied as an inhibitor may be inactive but metabolized to an active form. The well-known inhibitory effect of fluoro-acetate is apparently due to its metabolic conversion to fluorocitric acid which is an inhibitor of aconitase. There is evidence also that exchange reactions with the coenzyme may occur with some of the pyridine compounds; isonicotinic acid hydrazide has been found to undergo exchange with nicotinamide, and the corresponding DPN analogues have been isolated. Inhibition by structurally unrelated compounds occurs also as in the inhibition of invertase by certain basic dyes; sucrose and other sugars compete with the dye for the enzyme, suggesting that a variety of chemically different substances having appropriately distributed charges may compete for acidic or basic groups on the enzyme to produce inhibition of its activity.

The nature of the antibacterial activity of enzyme inhibitors cannot be sharply differentiated from the antibacterial effects of substances having utility as disinfectants. While the lethal action of disinfectant substances is a net effect, as pointed out earlier, probably the most important single effect, though not necessarily always the quantitatively predominant one, is inactivation of enzyme systems. Thus, the affinity of heavy metals for sulfhydryl groups, including those of enzymes, is well known, and the bisphenols, such as hexachlorophene, strongly inhibit succinoxidase, cytochrome oxidase, and lactic dehydrogenase; still other examples of this kind of effect of disinfectants have been noted above.

ANTIBIOTIC SUBSTANCES[16, 20, 24, 46, 115, 122]

The mutual relationships occurring among the microorganisms are analogous to those well known among other living organisms, ranging from synergistic and beneficial associations to antagonistic relationships. Synergism, the relation in which a group of two or more kinds of microorganism has capabilities greater than the sum of those of the members of the group considered separately, is apparent in many situations, as different as the cyclic transformations of organic matter in the soil, and the dual or multiple etiology of infectious diseases such as swine influenza or gaseous gangrene.

The antagonistic relationship is often apparent between saprophytic and parasitic pathogenic microorganisms. In a general way, most pathogenic microorganisms are closely adapted parasites, poorly equipped to survive the rigors of intense competition with mixed microbial flora outside the body of the host. This appears to be the basis of the ability of the typhoid bacillus, for example,

to survive for 2 to 3 weeks in sterile water, but only a few days in natural waters and even less in polluted waters. Similarly, the pathogenic microorganisms present in the bodies of persons dead of infectious disease, with the exception of the relatively rare spore-forming pathogens such as the anthrax bacillus, are unable to survive in the soil, and there is no hygienic objection to earth burial of such bodies.

Thus the microbial population, both animal and plant, of natural habitats such as soil and water, is determined by the mutual relationships among the members of the population as well as other environmental conditions. The pathogen appears as a newcomer, too poorly adapted to establish an ecological niche in such habitats, and is unable to survive.

The antagonistic relationship is often attributable to the elaboration of substances by one kind of microorganism which are toxic or inhibitory to other kinds. The blue-green pigment pyocyanin, a phenazine compound produced by *Pseudomonas aeruginosa* (*Ps. pyocyaneus*), is actively toxic for very many kinds of bacteria and was, in fact, known as early as 1860 as the chloroform-soluble substance giving color to the "blue pus" of pyogenic infections with this microorganism—and before bacteria were known to be etiologically related to disease.

Such antimicrobial substances, originating as products of metabolism of living organisms, are known as antibiotics.[9] Such substances, elaborated under natural conditions in environments such as soil, which contains enormous numbers of a wide variety of microorganisms, play a significant role in microbial ecology.[116] In retrospect, saprophytic microorganisms present in soil seem a self-evident possible source of antimicrobial substances affecting pathogenic bacteria with some specificity, but the first antibiotic applicable to the treatment of infectious disease, penicillin, was not discovered until 1929 by Fleming. Since different kinds of bacteria differed in their sensitivity to its growth-inhibiting activity, penicillin was regarded as useful only in the preparation of selective mediums for the primary isolation of bacteria resistant to it such as Hemophilus species. It was not until 1940 that the concept of selectivity of action was extended to include man, with the consequence that its lack of toxicity for man in concentrations effectively inhibiting the growth of pathogenic microorganisms *in vivo* led to its use as a chemotherapeutic agent.

It is a common experience of those examining large numbers of isolation plates to find colonies of microorganisms surrounded by zones of no growth of other kinds of microorganisms present in the culture. It is but a single step from such observations to the inoculation of nutrient medium in a petri dish with a test bacterium so that a uniform film of growth will develop, followed by a second inoculation with a rich source of varied microbial flora, commonly soil, by the streak or other dilution method to give isolated colonies. After incubation the culture is examined for halo-like zones of growth inhibition of the test organism about colonies growing up from the second inoculation. When such zones are observed, the appropriate colony is subcultured and the subculture tested for the presence of soluble growth-inhibiting activity for the test bacterium. The activity may be purified by various appropriate chemical methods and tested for both antimicrobial activity and toxicity to an experimental animal. If the toxicity is sufficiently low in relation to antimicrobial activity, it is tested for chemotherapeutic activity in experimental infections.

This procedure is relatively simple and so has been widely applied with the demonstration of many hundreds of kinds of antibiotic activity. Fungi, actinomycetes and molds, have been the most prolific source of antibiotics, although some are of bacterial origin. Unfortunately, the great majority of these are toxic, some highly toxic, to animals and are discarded from consideration as possible chemotherapeutic agents. Nevertheless, some 340 useful

and potentially useful antibiotics are described in a compilation,[93] of which some twenty-odd are in active use. All show a greater or lesser degree of specificity of action on different kinds of bacteria, often associated with the Gram staining reaction, and the range of kinds of bacteria sensitive to the action of an antibiotic is spoken of as its "spectrum" of activity. Of the many antibiotic substances, only the most common will be considered here.

Antibiotic Substances from Bacteria. A variety of antibiotics are formed by the aerobic sporulating bacteria of the genus Bacillus. The first of these to be studied as a chemotherapeutic agent was isolated by Dubos in 1939 from *B. brevis,* and found to be an alcohol-soluble, water-insoluble polypeptide which was named *tyrothricin.*

Further investigation made possible its separation into two components, *gramicidin* and *tyrocidin,* of somewhat different properties though both contain "unnatural" amino acids of the *d* series. Gramicidin is a large cyclopeptide containing relatively large amounts of tryptophan and is effective only on gram-positive bacteria. A closely similar substance, *gramicidin S,* is formed by a thermophilic variety of *B. brevis* which differs in that it has considerable activity against gram-negative bacteria and is a cyclopeptide hydrochloride with one free amino group, no free carboxyl and one hydrochloride residue, made up of one residue each of *l*-ornithine, *l*-proline, *l*-valine, *l*-leucine, and *d*-phenylalanine. Tyrocidin is active against both gram-positive and gram-negative bacteria *in vitro,* but its activity is almost completely inhibited by serum proteins. These antibiotics are surface-active substances, and their antibacterial activity is perhaps attributable to their destructive effect on the cell wall of the bacterium.

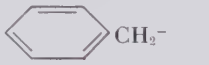
A number of antibiotic substances have been isolated from strains of *B. subtilis* which vary somewhat in their properties and antibiotic activity, though all appear to be polypeptide in nature. *Subtilin* is effective chiefly on gram-positive bacteria and certain of the acid-fast bacilli. It has low toxicity and is effective in the therapy of experimental infection of mice with pneumococci and guinea pigs with anthrax. *Bacitracin* is similar to subtilin and effective on gram-positive bacteria, but its utility is limited by its nephrotoxicity. *Bacillin* is highly active on both gram-positive and gram-negative bacteria *in vitro* and is only moderately toxic, but, like tyrocidin, its activity is destroyed by blood and it is completely ineffective as a chemotherapeutic agent. *Eumycin* does not affect gram-negative bacteria but has *in vitro* activity against the diphtheria and tubercle bacilli and some of the fungi. Its toxicity is low but its therapeutic efficiency is not known. *Licheniformin* resembles subtilin in its properties and is regarded by some workers as identical with it.

Other antibiotics from bacteria include *colicin* or *colistatin,* which are formed by coliform bacteria and inhibit gram-negative bacilli such as the dysentery bacilli. Some of the bacterial pigments show antibiotic activity, including *violacein* from *Chromobacter violaceum, phthiocol* formed by the tubercle bacillus, and *iodinin* from *Chromobacter iodinum.*

Antibiotics Produced by Higher Fungi. So far the most prolific source of chemotherapeutic antibiotics has been the higher fungi, that is, certain molds but especially the actinomycetes of the genus Streptomyces. These substances are relatively simple compounds of molecular weights of less than 1000.

Penicillin. Penicillin is not a single substance, but a group of closely related compounds differing from the parent β-lactam monobasic carboxylic acid with respect to the side chain. These substances are formed by *Penicillium notatum,* twenty-two other species of Penicillium, and seven species of Aspergillus. The six common naturally occurring forms are shown in the accompanying table. Two other penicillins are produced by biosynthesis. One is cephalosporin N, produced by a species of Cephalosporium, which has been shown to be (D-4-amino-4-carboxy-*n*-butyl)

Naturally Occurring Penicillins

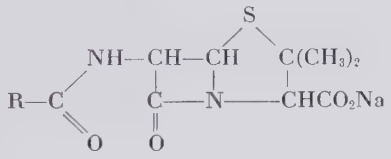
NAME	SYNONYM	SOURCE	R
penicillin F	penicillin I	*P. notatum* *P. chrysogenum*	$CH_3CH_2CH=CHCH_2^-$
dihydro F penicillin	gigantic acid	*A. giganteus*	$CH_3CH_2CH_2CH_2CH_2^-$
flavicin flavicidin	F type	*A. flavus*	$CH_3CH=CHCH_2CH_2^-$
penicillin G	penicillin II	*P. notatum* *P. chrysogenum*	
penicillin X	penicillin III	*P. notatum* *P. chrysogenum*	
penicillin K	penicillin IV	*P. notatum* *P. chrysogenum*	$CH_3CH_2CH_2CH_2CH_2CH_2CH_2^-$

penicillin. The other is penicillin V, phenoxymethyl penicillin, which is formed by *P. chrysogenum* when N-2-hydroxyethyl phenoxyacetamide is supplied as a precursor, which, in contrast to other penicillins, is stable as the free acid.

In addition, various relatively insoluble, and therefore less readily excreted, forms of penicillin have been prepared, including benethamine (N-benzyl-β-phenylethyl penicillin), hydrabamine (N,N′ dihydrobutyl ethylenediamine dibenzyl penicillin) and benzathine (Benzethacil, Bicillin) (N,N′ dibenzyl diethylenediamine penicillin G.). Of these various forms of penicillin, penicillin G (phenacetyl penicillin) is the one generally available as a stable salt because it is most readily produced on a large scale.

The alteration of penicillins produced by fermentation to give "synthetic" penicillins has resulted in the preparation of a number of compounds having valuable properties. The phenoxy compounds include α-phenoxymethyl, α-phenoxyethyl, and α-phenoxypropyl penicillins,[118] which are acid-resistant and can be given by mouth. A compound prepared from naturally formed 6-aminopenicillanic acid, 6[D(-)α-aminophenylacetamido] penicillanic acid (Penbritin), is also acid resistant and, in addition, has a considerably broader spectrum of activity than the other penicillins.[84, 100] Special interest has attached to sodium 6-(2,6-dimethoxybenzamido) penicillinate (Celbenin, methicillin, Staphcillin), because the alteration in its structure is sufficient that it is not destroyed by penicillinase and is, therefore, effective against penicillin-resistant staphylococci.[5, 14, 61, 85, 101]

Penicillin is produced by biosynthesis,[12] although it has been synthesized, not because of the complexity of the molecule, but because it is relatively unstable. The synthetic product is not yet competitive with that produced by fermentation.



penicillin

Penicillin is most active on gram-positive bacteria but some gram-negative forms such as the gonococcus and meningococcus are susceptible to it, as well as most spirochetes and the larger viruses of the psittacosis-lymphogranuloma group. It appears to act primarily as a bacteriostatic agent though high concentrations may be bactericidal. In general it is more active than the sulfonamides and less so *in vitro* than the triphenylmethane dyes.

Bacteria exposed to penicillin become swollen and fail to divide, and there is reason to believe that some processes

essential to cell division are affected. On treatment of bacteria with penicillin, the antibiotic is irreversibly bound to the bacterial cell. The formation of enzymes concerned with the assimilation of glutamic acid is interfered with, nonamino nitrogenous material appears in the suspending medium, and the ratio of ribonucleic acid phosphorus to deoxyribonucleic acid phosphorus decreases. The cell is apparently stripped of ribonucleic acid, and the synthesis of protein is disturbed with the accumulation of polypeptide that may or may not be a normal intermediate in that synthesis. The data are consistent with the assumption that penicillin interferes with the biosynthesis of cell wall substance of sensitive bacteria, a metabolic sequence not occurring in animal cells, and resembles in some respects the action of crystal violet.[26, 73]

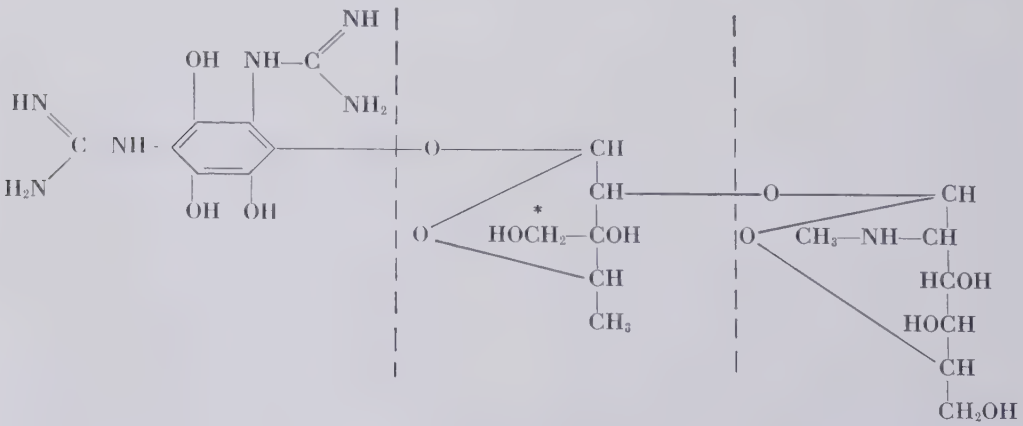
Penicillin is inactivated by an enzyme, penicillinase, which is produced by a wide variety of microorganisms, especially Bacillus species.[75]

Streptomycin. This antibiotic is formed by *Streptomyces griseus* and was isolated by Waksman in 1943. It is a basic glucoside and may be changed to dihydrostreptomycin by hydrogenation. The structural formula of dihydrostreptomycin is shown in 7, the starred carbon indicating the point at which hydrogen is added; in streptomycin this alcohol group is an aldehyde. These substances differ from penicillin in their activity in that they are effective on gram-negative bacteria and also on the tubercle bacillus. The susceptibility of bacteria to streptomycin is variable from strain to strain, and the sensitivity of the infecting organism, particularly in the case of green streptococci, should be tested. A serious drawback to the use of this antibiotic is the facility with which sensitive bacteria become resistant to it.

Streptomycin and dihydrostreptomycin do not differ in antibacterial activity from one another except that some strains of Salmonella are less sensitive to the dihydro compound. The dihydro form is preferable in the treatment of infectious disease because it is less toxic than streptomycin. The toxicity is manifested as an effect on the eighth nerve and vestibular apparatus and, following prolonged therapy, vertigo, tinnitus, and eighth nerve deafness (which may be irreversible) occur to a significant extent. The dihydro compound has somewhat less tendency to affect the vestibular apparatus.

Unlike penicillin, streptomycin affects the respiration of sensitive bacteria. It has been found by Umbreit and his coworkers that it inhibits the oxalacetate pyruvate condensation, thus preventing various compounds from entering into the terminal respiratory system. Citrate formation from oxalacetate and pyruvate is

7.



streptidine *streptose* *N-methyl-l-glucosamine*

dihydrostreptomycin

not inhibited, however, indicating that a pyruvate reaction not involving citrate is the one inhibited. The same results have been obtained with tissue homogenates. The oxalacetate-pyruvate condensation occurring in the mitochondria of tissue cells is not inhibited except after prolonged exposure to the antibiotic, and this has been taken to suggest that the specificity for the bacterial reaction, when it occurs *in vivo*, is a result of a differential permeability of host cells and mitochondria.

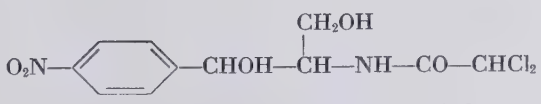
***Chloramphenicol* (*Chloromycetin*).** This antibiotic, formed by *Streptomyces venezuelae*, was described in 1947 by Ehrlich and his associates. Its structure has been determined, and it is now prepared by synthesis, the only antibiotic so prepared on a large scale. The structure is of some interest because aromatic nitro compounds and derivatives of dichloracetic acid are relatively rare in nature (8). This antibiotic, together with the tetracyclines, makes up the group of "broad spectrum antibiotics," *i.e.*, those that are effective against a wider variety of microorganisms than either penicillin or streptomycin. Chloramphenicol is effective not only against the gram-positive bacteria, but also the gram-negative bacteria and the typhoid bacillus in particular. It is also active against the rickettsiae and the viruses of the psittacosis-lymphogranuloma group, Its efficacy in typhoid fever is unique and it is the drug of choice in the treatment of this disease. It is, however, relatively ineffective in bacillary dysentery.

It differs from penicillin in that it does not inhibit the assimilation of glutamic acid, but it does inhibit the assimilation of ammonia and protein synthesis; the inhibition of protein synthesis has been shown directly and also may be inferred from the property chloramphenicol possesses in common with streptomycin, chlortetracycline, and oxytetracycline of a nonspecific inhibition of adaptive enzyme formation. This appears to be referable, in part at least, to a blocking of the synthesis of ribosomes, at the point of incorporation of amino acids in the formation of the 30S particle and that of the 100S particle.[83] It also inhibits esterase activity, both that of microbial origin and that from other sources; the inhibition of the esterase activity of tissue cells and mitrochondria is only partial, suggesting, as in the case of streptomycin and the oxalacetatepyruvate condensation, and explanation of *in vivo* specificity for the microorganism in this regard.

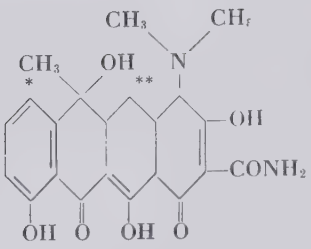
Chloramphenicol is relatively nontoxic by mouth and is effective by the oral route, for it is readily and almost completely absorbed from the gastrointestinal tract, and only occasionally nausea, vomiting, or diarrhea is produced. There have, however, been a number of reports of aplastic anemia following the administration of this antibiotic, perhaps to be expected since it is a nitro compound.

Tetracyclines. Chlortetracycline (Aureomycin), produced by *Streptomyces aureofaciens*, and oxytetracycline (Terramycin), produced by *Streptomyces rimosus*, were found independently, the former in 1948 by Duggar and his associates and the latter by Finlay and his associates in 1950. It soon became apparent that the two resembled one another closely, and it was subsequently shown that they are isomorphic tetracycline compounds, chlortetracycline containing non-ionic chlorine at the 16th carbon and oxytetracycline a hydroxy group on the 12th carbon. The parent compound, tetracycline (Achromycin,

8.



chloramphenicol



tetracycline

Chlortetracycline has a Cl attached at *, while oxytetracycline has a hydroxyl group attached at **.

Polycycline, Tetracyn, Stechlin, Panmycin), is obtainable by catalytic reduction of chlortetracycline and also by fermentation by a species of Streptomyces.

Chlortetracycline is distinguished by its golden-yellow color. The chloride is stable, but in neutral solution the antibiotic decomposes relatively rapidly at incubator temperatures. This makes for some difficulty in the microbiological assay of its activity, and cultures need to be observed after short periods of incubation. Its bacterial spectrum is closely similar to that of chloramphenicol, but it is not as effective in the treatment of typhoid fever.

The nature of its antibacterial activity is not clear. It may be inferred from the inhibition of adaptive enzyme formation that it exerts an inhibitory effect on protein synthesis. It has been found to inhibit phosphorylation, but not respiration, of mitochondria, and the phosphorylation of adenine nucleotides by orthophosphate is blocked, suggesting the possibility of interference with the Krebs cycle.

Chlortetracycline is relatively nontoxic on oral administration; untoward reactions are mild and include nausea, vomiting, and/or diarrhea. Parenteral inoculation is painful and results in an erythematous tender area at the site of intramuscular inoculation. In practice the requirement for parenteral administration is limited since the drug is well absorbed from the gastrointestinal tract.

Demethylchlortetracycline, having one less methyl group, reaches blood levels more slowly, but renal clearance is low, and blood levels tend to persist. It is reported to have equal, if not greater, antibacterial activity then the parent compound.[18, 89]

Oxytetracycline is a white substance which in neutral solution is more stable than chlortetracycline but resembles it closely in antibacterial activity, efficacy when given by mouth, and other factors. In inhibitory concentrations it blocks the utilization of ribonucleic acid reserves that are lost to the surrounding medium after depolymerization to mononucleotides. It also inhibits respiration in the presence of 4-carbon dicarboxylic acids, pyruvate, tricarboxylic acids, and α-ketoglutarate.

The parent compound, tetracycline, does not differ significantly from the chlor- and oxy-derivatives in antibacterial activity.

Chemical Classification.[1, 16, 68, 93] While the antibiotics are chemically diverse, most of them fall into three general groups, *viz.:* (1) those derivable from amino acids, including penicillin, chloramphenicol, bacitracin, polymyxin, and cycloserine; (2) those derivable from acetate, including the tetracyclines, griseofulvin, the erythromycin group of macrolides, and the conjugated polyenes, amphotericin B and nystatin; and (3) those derivable from sugars, including streptomycin, kanamycin, and neomycin.

The macrolide antibiotics are characterized by a lactone ring, a ketone function commonly an α-β unsaturated system, and a deoxyamino sugar containing a dimethyl amino group. Some of these are useful as esters formed by attachment of acyl groups to various hydroxyl groups. Thus the propionyl ester (as the lauryl sulfate salt) and the ethyl carbonate ester, esterified in the hydroxyl group of desosamine, of erythromycin have practical utility.

The polyene antibiotics, having the general structure $(CH{=}CH)_n$, also contain large lactone rings, but are set apart from the macrolides both chemically and biologically. They are active against a variety of fungi, including both yeasts and filamentous forms, but are inactive against bacteria or actinomycetes. Of

these, amphotericin B, a heptaene polyene, and nystatin, a diene-tetraene polyene, have been most useful in the treatment of a variety of fungous diseases (Chap. 32).

THE APPLICATION OF CHEMOTHERAPEUTIC AGENTS

Since the antimicrobial activity of the chemotherapeutic agents (not only the antibiotics but also sulfonamides and the synthetic compounds such as *p*-aminosalicylic acid and thiosemicarbazones) is primarily growth-inhibitory, it is assayed by growth-inhibition tests. Conversely, the relative sensitivity of kinds and strains of microorganisms is determined by test against standard concentrations of such substances.

Assay of Activity. The assay methods are of two general kinds, the tube method and the disc method. In the former, serial dilutions of the substance are prepared in a standard liquid culture medium, and the culture tubes (or flasks) are inoculated with a constant number of bacteria, incubated, and examined for growth. Thus the activity may be measured as the smallest amount of the active substance that prevents growth under the specified conditions. When large numbers of tests are to be carried out, as in the routine testing of bacterial sensitivity, the method is relatively time-consuming, and so is not often used.

The so-called disc method is based on the zone of growth inhibition surrounding a colony of antibiotic-producing microorganisms growing in culture in which the test microorganism is growing as a continuous film on the surface of the agar medium as described earlier. The first such method devised consisted of placing small, sterile, glazed ceramic or glass cylinders vertically on the surface of the uniformly inoculated agar medium and pipetting into them serial dilutions of the substance under test. The activity diffuses outward in the culture medium and, within limits, the activity of the preparation may be determined by comparison with the zone of growth inhibition produced by the standard. Six such cylinders may be appropriately arranged in the usual petri dish culture without overlap of inhibition zones. The standard on which such an assay is based is arbitrarily determined. The activity of penicillin, for example, was originally defined as the Oxford unit, a standard strain of Staphylococcus being used as the test microorganism. This was subsequently replaced by the International Unit which approximates the Oxford unit; the standard preparation is one of crystalline sodium penicillin G, containing 98.5 per cent of penicillin G. It contains 1670 IU per ml., and the IU is 0.5988 $\pm$ 0.5 per cent μg.[39] Similarly the IU of streptomycin is arbitrarily defined, but almost equivalent in activity to that of 1 μg of pure dihydrostreptomycin base,[37] and the oxytetracycline standard preparation contains 900 IU per mg.[38] Similar international standards have been defined for tetracycline[35] and for erythromycin.[36]

This kind of assay is not confined to the measurement of the activity of chemotherapeutic substances but has also been applied to other antimicrobial substances such as the quaternary ammonium compounds. In contrast to assays of the phenol coefficient type, it does not depend upon a sterility end point and measures growth-inhibitory rather than lethal activity.

Sensitivity Tests. The great bulk of such assays, however, is directed toward the measurement of the sensitivity of microorganisms isolated from disease conditions as a guide to effective chemotherapy. While certain generalizations may be made, *viz.*, gram-positive bacteria are sensitive to penicillin and gram-negative bacteria, with certain exceptions, are not, and the converse relation holds true with streptomycin, too much reliance cannot be placed upon them for two reasons. First, bacteria may become resistant to chemotherapeutic substances (Chap. 7) so that the sensitivity of a given strain may not be predicted with complete confidence. Second, some kinds

of bacteria, such as α-hemolytic streptococci, vary widely from strain to strain in their sensitivity to antibacterial chemotherapeutic agents. Testing for sensitivity of isolates has, then, become a routine diagnostic adjunct.

The sensitivity spectrum so determined depends upon the context from which it is taken. The usual spectrum of a given chemotherapeutic substance is commonly derived from stock cultures of bacteria, but that found with isolates from infectious processes may differ, usually in that resistant strains of bacteria "normally" sensitive to a given agent may be found. In a general way, such a shift is most pronounced in isolates from populations of hospitalized persons, and tends to be related to the kinds of chemotherapeutic agents used and the extent to which they are used. The utility of a given chemotherapeutic substance thus depends upon a number of factors, and the spectrum of its activity is more realistically given by the proportions of bacterial strains found to be sufficiently sensitive to it so that it has utility as a therapeutic agent. An illustration of such a practical spectrum of activity is given in the accompanying table.

The diagnostic sensitivity test. The sensitivity test applied for this purpose is considerably simplified by the use of filter paper discs impregnated with appropriate concentrations of the chemotherapeutic substances. This kind of test is recommended as an international standard,[127] and for routine tests with pathogenic bacteria (excluding tubercle bacilli) the following antibiotics should be used: erythromycin, novobiocin, vancomycin, bacitracin, penicillin, tetracycline, chloramphenicol, neomycin, streptomycin, and polymyxin. The discs are placed upon the surface of the agar culture medium uniformly inoculated with the test bacterium. There are many variations on this scheme, including cogwheel-shaped pieces of filter paper having the projections impregnated with different substances, circular pieces with impregnated projections toward the center, etc. In any case, two concentrations of the antibacterial substances are usually used, a "high" and a "low" concentration. For example, the concentrations of penicillin are 10 and 1.5 units, of tetracycline 25 and 4 units, erythromycin 10 and 1 unit, streptomycin 100 and 10μg, etc. The "high" concentration is intended to approximate the maximum blood levels of the substance attainable, but this is not always true. For instance, for technical reasons, sensitivity to streptomycin for therapeutic purposes is best shown by the indicated concentrations, although in this case the blood level attained is more nearly 10 μg than 100 μg because of toxicity.

After incubation the test is read as the diameter in millimeters of the zones of growth inhibition, but judgment must be used since the size of the zone of inhibition is also a function of the rate of diffusion of the activity into the culture medium. A high degree of sensitivity, for instance, is indicated by a maximal zone of inhibition around the "low" concentration even though that around the "high" concentration may not be larger because of the diffusion factor. This method of assay of bacterial sensitivity is widely applied and gives remarkably consistent results in view of its relative crudity.

Secondary Infections.[65] The microbial flora of man, and other animals, is diverse, and when an antimicrobial agent is administered for therapeutic purposes, there is an inevitable side effect on the normal flora due to the selective toxicity of the agent. In the normal individual the character of the microbial flora is determined by many factors, and of these a significant element is the interrelationships of the microorganisms present in, for example, the large bowel. In this case, in addition to the quantitatively predominant coliforms, other gram-negative bacilli such as Proteus, sporulating anaerobes such as *Cl. welchii*, α-hemolytic streptococci or enterococci, staphylococci, fungi such as yeasts, and filamentous forms as *Candida albicans* are present in small proportion. When an antimicrobial substance is introduced, this flora changes

Per Cent Sensitivity to Antibiotics of Representative Microorganisms Isolated from Infectious Processes*

MICROORGANISMS		ANTIBIOTICS											
Kind	Number of Strains	Tetracyclines	Penicillin	Streptomycin	Chloramphenicol	Erythromycin	Carbomycin	Novobiosin	Bacitracin	Neomycin	Sulfonamides	Furadantin	Polymyxin B
Staphylococcus													
Staph. aureus	895	55	48	62	79	76	72	88	67	62	59	96	22
Staph. albus	238	61	62	79	84	90	86	100	67	—	58	100	—
Streptococcus													
Str. fecalis	330	41	32	14	65	53	42	67	—	—	2	91	—
other α strep.	40	95	88	73	98	98	93	50	—	60	21	87	20
β-strep.	68	90	88	38	94	93	88	50	60	—	50	100	—
γ-strep.	16	94	75	69	100	94	94	—	—	—	40	100	—
Coliform bacilli													
E. coli types													
E. coli	645	37	—	75	62	—	—	50	—	80	30	85	69
E. intermedium	466	33	—	45	60	—	—	—	—	83	28	42	71
E. freundii	42	31	—	52	60	—	—	—	—	90	33	100	69
A. aerogenes	108	25	—	40	54	—	—	—	—	—	25	35	59
paracolon types													
coliform	45	40	—	84	62	—	—	—	—	73	67	89	65
intermedium	73	48	—	66	58	—	—	—	—	85	41	58	52
aerogenoides	26	35	—	61	65	—	—	—	—	77	69	33	65
Pseudomonas													
Ps. aeruginosa	157	10	—	23	1	—	—	—	—	17	27	7	46
other Pseudomonas	58	45	—	29	28	—	—	—	—	52	48	27	53
Proteus													
Pr. mirabilis	213	1	—	81	51	—	—	—	—	79	48	10	1
Pr. morgani	37	30	—	57	38	—	—	—	—	76	43	—	11
Pr. rettgeri	11	9	—	—	9	—	—	—	—	73	9	—	9
Pr. vulgaris	10	—	—	60	50	—	—	—	—	60	40	—	—
Salmonella species	12	58	—	83	92	—	—	—	—	75	42	—	25

* Isolated in the summer of 1957 in the Clinical Microbiology Laboratory of the University of Chicago Clinics. These data are not necessarily characteristic of nonhospitalized infections, those of other hospitals, or of this hospital at other times. (Compiled by Dr. R. S. Benham and Miss Isabelle Havens.)

Assay of the Antibiotic Sensitivity of Bacteria*

HIGH-CONCENTRATION DISCS: DIAMETER OF ZONE OF GROWTH INHIBITION IN MM.	LOW-CONCENTRATION DISCS: DIAMETER OF ZONE OF GROWTH INHIBITION IN MM.									
	11	12	13	14	15	16	17	18	19	20+
11	−									
12	−	−								
13	−	−	−							
14	−	−	−	−						
15	+	+	+	+	+					
16	+	+	+	+	+	+				
17	+	+	+	+	+	+	++			
18	+	+	+	+	+	++	++	++		
19	+	+	+	+	+	++	++	++	++	
20	+	+	+	+	++	++	++	++	++	+++
21	+	+	+	+	++	++	++	++	++	+++
22	+	+	+	+	++	++	++	++	++	+++
23	+	+	+	+	++	++	++	++	++	+++
24	+	+	+	+	++	++	++	++	++	+++
25	+	+	+	+	++	++	++	++	++	+++
26	+	+	+	+	++	++	++	++	++	+++
27	+	+	+	+	++	++	++	++	++	+++
28	+	+	+	+	++	++	++	++	++	+++
29	+	+	+	+	++	++	++	++	++	+++
30	+	+	+	++	++	++	++	++	+++	+++
31	+	+	+	++	++	++	++	++	+++	+++
32	+	+	+	++	++	++	++	++	+++	+++
33	+	+	+	++	++	++	++	+++	+++	+++
34	+	+	+	++	++	++	++	+++	+++	+++
35+	+	+	+	++	++	++	++	+++	+++	+++

− = resistant; + = slightly sensitive; ++ = moderately sensitive; +++ = sensitive.

* According to the evaluation method used in the Clinical Microbiology Laboratory of the University of Chicago.

in character with the emergence of minority forms that, when present in greatly increased proportions, assume some degree of pathogenicity. This effect is most commonly observed following oral administration of broad spectrum antibiotics but may also occur when other antimicrobial substances and other routes of administration are used.

Thus secondary infections may occur which are quite unrelated to the disease condition for which therapy was initiated. There are three kinds of more common consequences. First moniliasis or candidiasis (Chap. 32) may develop by uninhibited, or possibly stimulated, proliferation of the fungus *C. albicans* with consequences ranging from, for instance, local irritation in the bowel, anus, or vagina, to a generalized, sometimes fatal, infection, especially in young children. Second, with selective inhibition of other components of the intestinal flora, staphylococci, often resistant forms, may become predominant to produce diarrheal disease and occasionally fatal staphylococcal toxemia. Third, when the growth of coliforms and enterococci is markedly depressed, forms such as Proteus and Pseudomonas may grow freely and invade the tissues to produce infections of the kidneys or urinary bladder. Still other effects may be produced by intensive antibiotic therapy. For example, the bacterial flora of the mouth and throat often become predominantly coliform in character, with coliform throat cultures not uncommonly encountered in the diagnostic laboratory, and it is believed by some that some strains of coliform bacilli, especially hemolytic forms, may produce inflammatory changes in the throat.

The over-all effect of the general use of chemotherapeutic agents is illustrated in compiled data from the Boston City

Hospital from 1935 to 1957.[19] Staphylococcal infections of the blood and meninges increased greatly, and there has been a coincident enormous increase in bacteremia and meningitis due to gram-negative bacilli, *viz.*, Proteus, Klebsiella (coliform) and Pseudomonas. The mortality from bacteremia decreased from 1935 to 1947, but thereafter rose rapidly until by 1957 it was 50 per cent higher than in 1935.

Combined Therapy.[62, 105] In view of the selective action of antimicrobial substances, their administration in combination is inevitable, both to broaden the antimicrobial activity of the preparation in the absence of precise diagnosis of the etiological agent, in treatment of mixed infections, and minimize the secondary infections occurring during intensive therapy described above and to reduce the probability of the development of resistance by the microorganism (Chap. 7).

Various combinations, sometimes mixtures and sometimes compounds, have been prepared. Among the mixtures, the combination of streptomycin or isoniazid with *p*-aminosalicylic acid is almost routinely used in the treatment of tuberculosis; this is something of a unique situation in that the disease requires prolonged therapy which favors the development of bacterial resistance, and because of the facility with which the bacilli become resistant to streptomycin and, to a lesser extent, to isoniazid. Other combinations include the admixture of antifungal substances such as candicidin, fungicidin, or undecylenic acid with the broad spectrum antibiotics, especially the tetracyclines, to minimize fungal overgrowth during therapy, and various combinations of antibiotics such as tetracycline plus nystatin (Mysteclin) or plus oleandomycin (Sigmamycin, Signemycin).

Compounds of antibacterial substances include: streptomycyclidene isonicotinyl hydrazide sulfate (Streptohydrazid); Streptobine, a stable salt containing 48 per cent dihydrostreptomycin, 34 per cent isoniazid, and 18 per cent pyruvic acid; Hiconstarch, a polymer prepared from periodate-oxidized potato starch by condensation with equimolar proportions of isoniazid and *p*-aminobenzalthiosemicarbazone; a compound of isoniazid and *p*-aminosalicylic acid (Pasiniazide, Dipasic); isonicotinyl hydrazone benzaldehyde metasulfonic acid (G605, Sulfoniazide); etc. The value of such preparations is not established.

Synergism and antagonism. Contrary to what might be supposed, the effect of combination of two substances demonstrable with a single species of microorganism susceptible to both is not necessarily an additive one. This phenomenon has been studied by a number of workers, especially by Jawetz and his colleagues[45, 47] and by Klein and his coworkers.[60] Klein has summarized the kinds of effect produced by drug combinations both *in vitro* and *in vivo* as follows:

> (1) A synergistic effect may be defined as that in which the activity of two drugs in combination is greater than that obtained by doubling the concentration of either alone.
> (2) An additive effect is that in which the antibacterial activity of the combination is greater than that of either drug alone, but less than that obtained by doubling the concentration of either.
> (3) The effect is one of interference when the antibacterial activity of the combined drugs is not greater than that of either drug alone.
> (4) Two drugs are said to be antagonistic when the antibacterial activity of the combination is less than that obtained with either drug alone.

Effects falling into one or the other of the above categories have been observed with mixtures of sulfonamides with antibiotics and with mixtures of different antibiotics but are sometimes difficult to interpret. For example, one of the most widely tested combinations is that of streptomycin with *p*-aminosalicylic acid in the therapy of tuberculosis; this combination appears to be invariably synergistic. The combination of penicillin and sulfonamides, however, is initially antagonistic, but this is transitory and the effect eventually becomes synergistic.

Jawetz has generalized the interrela-

tionships among the antibiotics by placing them into two groups: the first is made up of penicillin, streptomycin, bacitracin, and neomycin; and the second of Aureomycin, chloramphenicol, and Terramycin. He has found that members of the first group are often synergistic with each other and never antagonistic, while pairs of the second group show only an additive effect. The consequence of combining antimicrobial substances of different groups is variable and dependent upon the sensitivity of the microorganism to the drug in the first group; if it is highly sensitive, the second antimicrobial substance will interfere, but if it is relatively resistant the combination may be synergistic. He has suggested the following possible explanations:

(1) The two antimicrobial substances combine chemically to give a more active compound.
(2) One antimicrobial substance is effective on those bacteria that are resistant to the other.
(3) One antimicrobial substance affects actively multiplying bacteria and the other viable but nonreproducing microorganisms.
(4) One antimicrobial substance potentiates the other indirectly, for example by decreasing the permeability of the cell wall.
(5) The combination of antimicrobial substances simultaneously blocks more than one metabolic pathway, preventing a facile shift by the microorganism in the presence of a single antimicrobial substance.

Of these, the first is quite unlikely on the basis of present evidence. The second is probably the explanation of the PAS-streptomycin synergism in the treatment of tuberculosis. The third is probably the explanation of the behavior of sulfonamide-penicillin combinations, *i.e.*, the sulfonamides are primarily bacteriostatic and exert an immediate effect, while penicillin is effective only on actively multiplying microorganisms. Evidence has been presented in support of the fifth possibility in the case of penicillin-streptomycin and Terramycin-bacitracin combinations.

Klein has taken the view, and supported it by strong experimental evidence, that synergism occurs only when the microorganism tends to become resistant and is related to the inhibition by the second drug of resistant microorganisms surviving the action of the first. It follows that when resistance does not develop with some facility, synergism commonly does not occur, and some degree of antagonism may be observed. The consequences of combining drugs are dependent upon the drugs, upon the strain of microorganism, and upon the conditions under which the organism is subjected to the action of the drugs. On the basis of present information, then, the activity of combinations of drugs is more or less unpredictable, and this is in accord with the observations of many workers. The considerable uncertainty as to the indication for combined therapy may, then, be resolved by the requirement that the microorganism show a significant increase in resistance to the chemotherapeutic agent, either promptly in acute disease or ultimately in chronic infections. Obviously, each of the combined drugs must have some activity against the microorganism, and they must differ from one another in their modes of action in such a way that cross-resistance does not develop. It is quite clear that as yet no rationale has been formulated for combined therapy of infections with microorganisms that do not become resistant with reasonable facility, *e.g.*, infections with pneumococci, *Str. pyogenes*, and others, and the fundamental distinction must be made between infections in which resistance is and is not a factor.

Intracellular Infections.[30, 34, 40, 64, 96] While the mature virus particle shows substantially the same susceptibility to the lethal, or inactivating, effects of disinfectants as the bacteria, the diseases of viral etiology are, with the exception of those caused by agents of the psittacosis-lymphogranuloma group, unaffected by the substances which are effective in the treatment of diseases of bacterial etiology. This appears to be a consequence of two characteristics of the viral agents, *viz.*, replication of the virus entirely within the host cell and the failure of the mature virus particle to show any evidence of independent metabolic activity.

Considering the sequence of events in the replication of a virus, there would appear to be three points of attack, *viz.*, inactivation of the mature virus particle prior to its entry into the host cell, interference with the initial stage of adsorption on the host cell, and inhibition of the intracellular synthesis of virus substance. As regards the first, substances whose activity is primarily one of enzyme inhibition are without effect as might be anticipated in view of the lack of independent viral metabolism. Disinfectants which do inactivate are, of course, toxic to the cells and tissues of the host. In connection with the second, there is some prophylactic effect of substances which interfere with initial adsorption of the virus particle, such as that of RDE in preventing the adsorption of influenza virus as described elsewhere (Chap. 4), but this is not a therapeutic effect and does not prevent successive developmental cycles in infected animals.

Finally, the intracellular phase of viral proliferation poses unique difficulties. One of these is the protection afforded by the host cell. The differential toxicity of antimicrobial substances is in part a consequence of differences in the permeability of the bacterial and host cells. The plasma membrane of the host cell is relatively impermeable, and the mitochondria even more so, to substances such as streptomycin, and there are possibly still other factors such as the presence of intracellular inhibitors. The protection afforded the intracellular parasite is illustrated by the resistance for periods of 24 to 48 hours of phagocytosed gonococci in culture fibroblasts or HeLa cells to concentrations of penicillin to which the free bacteria are sensitive.[113] It is not unlikely that protection of the intracellular parasite may be a significant factor in the unsatisfactory state of chemotherapy of certain bacterial diseases such as brucellosis as well as in diseases of viral etiology.

The efficacy of chemotherapeutic substances may also lie in part in their specific effect on a metabolic sequence of the bacterium which is not present in the host cell. In viral replication, however, the metabolic systems of the host cell are used. Nevertheless, it is possible that the host cell synthetic processes are sufficiently distorted that they may be preferentially affected, but this is not known. Further, it is possible that differences in reaction rates might lead to a differential toxicity, *i.e.*, a much greater effect on rapid viral replication than on the endogenous metabolism of the host cell.

This last has been explored to a certain extent. It will be recalled that the replication of viral substances involves synthesis of both viral protein and viral nucleic acid as seemingly somewhat different processes, and of which the latter is the more important in that it is essential to the production of complete virus. Attention has, then, been directed toward the activity of nucleic acid analogues in an effort to interfere more or less selectively with the synthesis of viral nucleic acid. A number of benzimidazole compounds have, for instance, been found to have appreciable chemotherapeutic effect under experimental conditions but, on continued administration, are toxic to the infected animal. It is unfortunate that the nature of the autointerference effect is not better understood for it results in the failure to form viral nucleic acid though viral protein is synthesized, with the result that incomplete or noninfective virus is produced by the host cell. In any case, effective chemotherapy of viral diseases has not yet been achieved.

As indicated above, diseases produced by agents of the psittacosis-lymphogranuloma group are an exception in that they may be effectively treated with a number of antibiotics. Similarly, diseases of rickettsial etiology are susceptible to treatment with antibiotics. These two groups of agents are, however, dissimilar to the viruses. Studies on the psittacosis viruses have suggested that a part of their developmental cycle may be extracellular, and there is evidence that they contain at least parts of coenzymes. The rickettsiae proliferate in dying, rather

than actively metabolizing, host cells and, in addition, show some small independent metabolic activity.

Other Applications. A number of the antimicrobial substances have useful applications other than in the treatment of infectious disease. The most important of these are the use of antibiotics as feed supplements and in food preservation. In the first instance it is established that the inclusion of small amounts (2 to 50 ppm) of antibiotics in animal feeds results in more rapid growth of domestic meat animals, increased hatchability of fertile eggs, and other indications of an enhanced nutrition, and such supplemented feeds are widely used. The nature of this effect is not clear.[102] It is thought by some to be a result of increased vitamin synthesis by the intestinal flora. The traces of antibiotics persisting in foods of such animal origin are apparently not significant, though there may be sufficient activity in eggs to prevent the growth of bacteria for experimental purposes in the embryonated hen's egg. There is also evidence that antibiotic-resistant strains of pathogens such as Salmonella may develop in birds fed such supplements.

The treatment of freshly dressed meats such as fish and fowl in dilute solutions of broad spectrum antibiotics has a preservative effect such that they stay fresh appreciably longer, and these substances may well have broad application in food preservation.[11, 17, 106] Chlortetracycline has been approved for this purpose in a concentration of 7 ppm; it is destroyed when the food is cooked.

REFERENCES

1. Abraham, E. P., and Newton, G. G. F.: Chemistry and classification of antibiotics. Brit. Med. J., 1960, **ii**:3–10.
2. Allen, M. B.: The dynamic nature of thermophily. J. Gen. Physiol., 1950, **33**:205–214.
3. Bellamy, W. D.: Preservation of foods and drugs by ionizing radiation. Adv. Appl. Microbiol., 1959, **1**:49–73.
4. Bennett, E. O.: Factors affecting the antimicrobial activity of phenols. Adv. Appl. Microbiol., 1959, **1**:123–140.
5. Brown, D. M., and Acred, P.: Chemotherapeutic studies on a new antibiotic—BRL 1241. Lancet, 1960, **ii**:568–569.
6. Bruch, C. W.: Gaseous sterilization. Ann. Rev. Microbiol., 1961, **15**:245–262.
7. Bruch, C. W.: Decontamination of enclosed spaces with β-propiolactone vapor. Amer. J. Hyg., 1961, **73**:1–9.
8. Bruhin, H., Bühlmann, X., Vischer, W. A., and Lammers, T.: Sterilisation mit Äthylenoxyd unter besonderer Berücksichtigung der Anwendung bei Kunstoffen. Schweiz. med. Woch., 1961, **91**:607–613; 635–639.
9. Bu'Lock, J. D.: Intermediary metabolism and antibiotic synthesis. Adv. Appl. Microbiol., 1961, **3**:293–342.
10. Dawson, F. W., Janssen, R. J., and Hoffman, R. K.: Virucidal activity of β-propiolactone vapor. II. Effect on the etiological agents of smallpox, yellow fever, psittacosis and Q fever. Appl. Microbiol., 1960, **8**:39–41.
11. Deatherage, F. E.: Use of antibiotics in the preservation of meats and other food products. Amer. J. Pub. Health, 1957, **47**:594–600.
12. Demain, A. L.: The mechanism of penicillin biosynthesis. Adv. Appl. Microbiol., 1959, **1**: 23–47.
13. Domagk, G.: Entwicklungslinien chemotherapeutischer Forschung. Klin. Woch., 1951, **29**:139–154.
14. Douthwaite, A. H., and Trafford, J. A. P.: A new synthetic penicillin. Brit. Med. J., 1960, **ii**:687–690.
15. Dulbecco, R.: Photoreactivation. In Radiation Biology, vol. 2, pp. 455–486. McGraw Hill, New York. 1955.
16. Eagle, H., and Saz, A. K.: Antibiotics. Ann. Rev. Microbiol., 1955, **9**:173–226.
17. Farber, L.: Antibiotics in food preservation. Ann. Rev. Microbiol., 1959, **13**:125–140.
18. Finland, M., and Garrod, L. P.: Demethylchlortetracycline. Brit. Med. J., 1960, **ii**:959–963.
19. Finland, M., Jones, W. F., Jr., and Barnes, M. W.: Occurrence of serious bacterial infections since introduction of antibacterial agents. J. Amer. Med. Assn., 1959, **170**:2188–2197.
20. Finland, M., and Savage, G. M. (eds.): Antimicrobial Agents and Chemotherapy 1961. American Society for Microbiology, Detroit. 1962.
21. Freeman, M. A. R., and Barwell, C. F.: Ethylene oxide sterilization in hospital practice. J. Hyg., 1960, **58**:337–345.
22. Fry, R. M., and Graves, R. I.: The survival of bacteria during and after drying. J. Hyg., 1951, **49**:220–246.
23. Gard, S.: Theoretical considerations in the inactivation of viruses by chemical means. Ann. N. Y. Acad. Sci., 1960, **83**:638–648.
24. Goldberg, H. S.: Antibiotics: Their Chemistry and Non-medical Uses. D. Van Nostrand, Princeton. 1959.
25. Goodlow, R. J., and Leonard, F. A.: Viability and infectivity of microorganisms in experimental airborne infection. Bact. Rev., 1961, **25**:182–187.
26. Hahn, F. E., and Ciak, J.: Penicillin-induced lysis of *Escherichia coli*. Science, 1957, **125**: 119–120.
27. Harris, R. J. C.: Biological Applications of Freezing and Drying. Academic Press, New York. 1954.

28. Hawking, F., and Lawrence, J. S.: The Sulphonamides. Grune and Stratton, New York. 1951.
29. Heckly, R. J.: Preservation of bacteria by lyophilization. Adv. Appl. Microbiol., 1961, **3**:1–76.
30. Herrmann, E. C.: The detection, assay and evaluation of antiviral drugs. Progress Med. Virol., 1961, **3**:158–192.
31. Hesselberg, I., Enell, H., and Sievers, O.: Freeze-drying experiments with Swedish BCG vaccine. Acta Path. Microbiol. Scand., 1958, **43**:162–170.
32. Hoffman, R. K., and Warshowsky, B.: β-propiolactone vapor as a disinfectant. Appl. Microbiol., 1958, **6**:358–362.
33. Horsfall, F. L., Jr., and Tamm, I.: Chemotherapy of viral and rickettsial diseases. Ann. Rev. Microbiol., 1957, **11**:339–370.
34. Humphrey, A. E.: Air sterilization. Adv. Appl. Microbiol., 1960, **2**:302–311.
35. Humphrey, J. H., Lightbrown, J. W., and Mussett, M. V.: International standard for tetracycline. Bull. W.H.O., 1957, **17**:521–526.
36. Humphrey, J. H., Lightbrown, J. W., and Mussett, M. V.: International standard for erythromycin. Bull. W.H.O., 1957, **17**:527–535.
37. Humphrey, J. H., Lightbrown, J. W., Mussett, M. V., and Perry, W. L. M.: The international standard for dihydrostreptomycin. Bull. W.H.O., 1954, **10**:901–909.
38. Humphrey, J. H., Lightbrown, J. W., Mussett, M. V., and Perry, W. L. M.: The international standard for oxytetracycline. Bull. W.H.O., 1955, **13**:903–915.
39. Humphrey, J. H., Mussett, M. V., and Perry, W. L. M.: The second international standard for penicillin. Bull. W.H.O., 1953, **9**:15–28.
40. Hurst, E. W.: Approaches to the chemotherapy of virus diseases. J. Pharmacy Pharmacol., 1957, **9**:273–292.
41. Hurst, A., Stuttard, L. W., and Woodroffe, R. C. S.: Disinfectants for use in bar-soaps. J. Hyg., 1960, **58**:159–176.
42. Hutchinson, F.: Molecular basis for action of ionizing radiations. Science, 1961, **134**:533–538.
43. Irwin, J. O.: The distribution of the logarithm of survival times when the true law is exponential. J. Hyg., 1942, **42**:328–333.
44. Isaacs, A.: Particle counts and infectivity for animal viruses. Adv. Virus Res., 1957, **4**:112–158.
45. Jawetz, E.: Antibiotic synergism and antagonism. Review of experimental evidence. Arch. Intern. Med., 1952, **90**:301–309.
46. Jawetz, E.: Antimicrobial chemotherapy. Ann. Rev. Microbiol., 1956, **10**:85–114.
47. Jawetz, E., and Gunnison, J. B.: Antibiotic synergism and antagonism: an assessment of the problem. Pharmacol. Rev., 1953, **5**:175–192.
48. Jordan, R. C., and Jacobs, S. E.: Studies on the dynamics of disinfection. I. New data on the reaction between phenol and *Bact. coli* using an improved technique together with an analysis of the distribution of resistance amongst the cells of the bacterial populations studied. J. Hyg., 1944, **43**:275–289.
49. Jordan, R. C., and Jacobs, S. E.: Studies on the dynamics of disinfection. II. The calculation of the concentration exponent for phenol at 35°C with *Bact. coli* as test organism. J. Hyg., 1944, **43**:363–369.
50. Jordan, R. C., and Jacobs, S. E..: Studies on the dynamics of disinfection. III. The reaction between phenol and *Bact. coli:* The effect of temperature and concentration with a detailed analysis of the reaction velocity. J. Hyg., 1945, **44**:210–220.
51. Jordan, R. C., and Jacobs, S. E.: Studies on the dynamics ot disinfection. IV. The reaction between phenol and *Bact. coli:* The true shape of the probit-log survival time curve. Ann. Appl. Biol., 1945, **32**:221–229.
52. Jordan, R. C., and Jacobs, S. E.: Studies on the dynamics of disinfection. V. The temperature coefficient of the reaction between phenol and *Bact. coli* derived from data obtained by an improved technique. J. Hyg., 1946, **44**:243–248.
53. Jordan, R. C., and Jacobs, S. E.: Studies on the dynamics of disinfection. VI. Calculation of a new and constant temperature coefficient for the reaction between phenol and *Bact. coli.* J. Hyg., 1946, **44**:249–255.
54. Jordan, R. C., and Jacobs, S. E.: Studies on the dynamics of disinfection. VII. The reaction between phenol and *Bact. coli:* The effect of temperature on the usually accepted concentration exponent and the calculation of a more satisfactory exponent based on theoretical considerations. J. Hyg., 1946, **44**:421–429.
55. Kelly, S., and Sanderson, W. W.: Chlorination of poliovirus. Science, 1957, **126**:560–561.
56. Kempe, L. L.: Sterilization of media for biochemical processes. Adv. Appl. Microbiol., 1960, **2**:313–319.
57. Kethley, T. W., Fincher, E. L., and Cown, W. B.: A system for the evaluation of aerial disinfectants. Appl. Microbiol., 1956, **4**:237–243.
58. Kimball, R. F.: Nongenetic effects of radiation on microorganisms. Ann. Rev. Microbiol., 1957, **11**:199–220.
59. Kleczkowski, A.: Effects of non-ionizing radiations on viruses. Adv. Virus Res., 1957, **4**: 191–220.
60. Klein, M., and Schoor, S. E.: The role of bacterial resistance in antibiotic synergism and antagonism. J. Bact., 1953, **65**:454–465.
61. Knox, R.: A new penicillin (BRL 1241) active against penicillin-resistant staphylococci. Brit. Med. J., 1960, **ii**:690–693.
62. Lacey, B. W.: Mechanisms of chemotherapeutic synergy. Society for General Microbiology, Symposium No. 8, pp. 247–287. Cambridge University Press, 1958.
63. Lowbury, E. J. L., Lilly, H. A., and Bull, J. P.: Disinfection of the skin of operation sites. Brit. Med. J., 1960, **ii**:1039–1044.
64. Mathews, R. E. F., and Smith, J. D.: The chemotherapy of viruses. Adv. Virus Res., 1955, **3**:49–148.
65. McCoy, E.: Changes in the host flora induced by chemotherapeutic agents. Ann. Rev. Microbiol., 1954, **8**:257–272.
66. Meryman, H. T.: Mechanics of freezing in living cells and tissues. Science, 1956, **124**:515–521.
67. Mortimer, R. K., and Beam, C. A.: Cellular radiobiology. Ann. Rev. Nuclear Sci., 1955, **5**:327–368.
68. Newton, G. G. F., and Abraham, E. P.: Some chemical and medical aspects of the antibiotics. J. Pharmacy Pharmacol., 1958, **10**: 401–426.

69. Niven, C. F., Jr.: Microbiological aspects of radiation preservation of food. Ann. Rev. Microbiol., 1958, **12:**507–524.
70. von Oettingen, W. F.: Phenol and its derivatives: The relation between their chemical constitution and their effect on the organism. Nat. Inst. Health Bull. No. 190, 1949.
71. Patt, H. M.: Biochemical aspects of basic mechanisms in radiobiology. National Academy of Sciences, Nat. Res. Council Nuclear Sci. Series, No. 17, 1954.
72. Patt, H. M., and Powers, E. L.: Cellular aspects of basic mechanisms in radiobiology. National Academy of Sciences, Nat. Res. Council Nuclear Sci. Series, No. 18, 1955.
73. Park, J. T., and Strominger, J. L.: Mode of action of penicillin. Biochemical basis for the mechanism of action of penicillin and for its selective activity. Science, 1957, **125:** 99–101.
74. Phillips, C. R., and Warshowsky, B.: Chemical disinfectants. Ann. Rev. Microbiol., 1958, **12:**525–550.
75. Pollock, M. R., Torriani, A.-M., and Tridgell, E. J.: Crystalline bacterial penicillinase. Biochem. J., 1956, **62:**387–391.
76. Pollard, E. C.: Theory of the physical means of the inactivation of viruses. Ann. N. Y. Acad. Sci., 1960, **83:**654–660.
77. Pollard, E. C.: The action of ionizing radiation on viruses. Adv. Virus Res., 1954, **2:**109–151.
78. Puck, T. T.: The mechanism of aerial disinfection by glycols and other chemical agents. I. Demonstration that the germicidal action occurs through the agency of the vapor phase. J. Exptl. Med., 1947, **85:**729–740.
79. Puck, T. T.: The mechanism of aerial disinfection by glycols and other chemical agents. II. An analysis of the factors governing the efficiency of chemical disinfection of the air. J. Exptl. Med., 1947, **85:**741–757.
80. Quastel, J. H.: Action of drugs on enzyme systems. In Gaebler, O. H. (ed.): Enzymes: Units of Biological Structure and Function, pp. 523–535. Academic Press, New York. 1956.
81. Rahn, O.: Physical methods of sterilization of microorganisms. Bact. Rev., 1945, **9:**1–47.
82. Reddish, G. F. (ed.): Antiseptics, Disinfectants, Fungicides and Chemical and Physical Sterilization. Lea and Febiger, Philadelphia. 2nd ed., 1957.
83. Roberts, R. B., McQuillen, K., and Roberts, I. Z.: Biosynthetic aspects of metabolism. Ann. Rev. Microbiol., 1959, **13:**1–48.
84. Rolinson, G. N., and Stevens, S.: Microbiological studies on a new broad-spectrum penicillin, "Penbritin." Brit. Med. J., 1961, **ii:**191–196.
85. Rolinson, G. N., et al.: Bacteriological studies on a new penicillin—BRL 1241. Lancet, 1960, **ii:**564–567.
86. Rosenthal, S. R., and Williston, E. H.: Factors influencing the activity of BCG in the process of freeze drying. Acta Tuberc. Scand., 1960, **38:**140–152.
87. Salle, A. J., McOmie, W. A., and Schechmeister, I. L.: The evaluation of a group of germicides by the tissue culture technique. J. Bact., 1939, **37:**639–646.
88. Shive, W.: Biological activities of metabolite analogues. Ann. Rev. Microbiol., 1952, **6:** 437–466.
89. Siegenthaler, W., Brovelli, L., Stutz, R., and Wiesmann, E.: Klinische und experimentelle Untersuchungen mit Demethylchlor-Tetrazyklin, einem neuen Breitspektrum-Antibioticum. Schweiz. med. Woch., 1960, **90:** 1315–1321.
90. Sizer, I. W.: Chemical aspects of enzyme inhibition. Science, 1957, **125:**54–59.
91. Sparrow, A. H., and Forro, F.: Cellular radiobiology. Ann. Rev Nuclear Sci., 1953, **3:**339–368.
92. Spaulding, E. H., and Bondi, A., Jr.: The evaluation of germicidal agents by an infection-prevention-toxicity method. J. Infect. Dis., 1947, **80:**194–200.
93. Spector, W. S.: Handbook of Toxicology. vol. II. Antibiotics. W. B. Saunders Company, Philadelphia. 1957.
94. Spicer, S.: A study of the biological characteristics of type III pneumococcus, cultivated at temperatures below 37°C. J. Bact., 1940, **39:** 517–526.
95. Spiner, D. R., and Hoffman, R. K.: Method for disinfecting large enclosures with β-propiolactone vapor. Appl. Microbiol., 1960, **8:**152–155.
96. Stachelin, M.: Experimental and biochemical basis for a chemoprophylaxis and chemotherapy of virus infections. Progress Med. Virol., 1959, **2:**1–42.
97. Stedman, R. L., Kravitz, E., and King, J. D.: Studies on cell surface-germicide and enzyme-germicide reactions and their contribution to the lethal effect. J. Bact., 1957, **73:** 655–660.
98. Stern, R. M., and Frazier, W. C.: Physiological characteristics of lactic acid bacteria near the maximum growth temperature. I. Growth and acid production. J. Bact., 1941, **42:**479–499.
99. Stern, R. M., and Frazier, W. C.: Physiological characteristics of lactic acid bacteria near the maximum growth temperature. II. Studies on respiration. J. Bact., 1941, **42:**501–512.
100. Stewart, G. T., Coles, H. M., Nixon, H. H., and Holt, R. J.: "Penbritin": an oral penicillin with broad-spectrum activity. Brit. Med. J., 1961, **ii:**200–206.
101. Stewart, G. T., Harrison, P. M., and Holt, R. J.: Microbiological studies on sodium 6-(2, 5-dimethoxybenzamide) penicillinate monohydrate (BRL 1241) in vitro and in patients. Brit. Med. J., 1960, **ii:**694–699.
102. Stokstad, E. L. R.: Antibiotics in animal nutrition. Physiol. Rev., 1954, **34:**25–51.
103. Sykes, G.: Disinfection. A critical review of methods and mechanisms. J. Pharmacy Pharmacol., 1955, **7:**561–579.
104. Sykes, G.: Disinfection and Sterilization: Theory and Practice. D. Van Nostrand, Princeton. 1958.
105. Symposium: Discussion on synergy in chemotherapy. Proc. Roy. Soc. Med., 1956, **49:**871–882.
106. Symposium: Proceedings First International Conference on the use of antibiotics in agriculture. National Academy of Sciences, Nat. Res. Council, Publication No. 397, 1956.
107. Symposium: Second conference on sulfonamides. Ann. N. Y. Acad. Sci., 1957, **69:** 377–524.
108. Symposium: The strategy of chemotherapy. Society for General Microbiology, Sympo-

sium No. 8. Cambridge University Press, 1958.
109. Symposium: Recent contributions to antibacterial therapy. Ann. N. Y. Acad. Sci., 1959, **82:**1–190.
110. Symposium: Inactivation of viruses. Ann. N .Y. Acad. Sci., 1960, **83:**513–760.
111. Symposium: Freezing and drying of biological materials. Ann. N. Y. Acad. Sci., 1960, **85:** 501–734.
112 Symposium: Symposium on methods for the evaluation of the antibacterial activity of surface active compounds. J. Appl. Bact., 1960, **23:**318–371.
113. Thayer, J. D., Perry, M. I., Magnuson, H. J., and Garson, W.: Failure of penicillin to kill phagocytosed *Neisseria gonorrhoeae*. Antibiotics and Chemotherapy, 1957, **7:**311–314.
114. Timm, E. A., McLean, I. W., Jr., Kupsky, C. H., and Hook, A. E.: The nature of the formalin inactivation of poliomyelitis virus. J. Immunol., 1956, **77:**444–452.
115. Verwey, W. F.: Newer antibiotics. Ann. Rev. Microbiol., 1959, **13:**177–190.
116. Waksman, S. A.: The role of antibiotics in nature. Perspect. Biol. Med., 1961, **4:**271–287.
117. Weinberg, E. D.: The mutual effects of antimicrobial compounds and metallic cations. Bact. Rev., 1957, **21:**46–68.
118. Williamson, G. M., Morrison, J. K., and Stevens, K. J.: A new synthetic penicillin PA-248. Lancet, 1961, **i:**847–850.
119. Withell, E. R.: The significance of the variation in shape of time-survivor curves. J. Hyg., 1942, **42:**124–183.
120. Withell, E. R.: The value of bactericides. J. Hyg., 1942, **42:**339–353.
121. Witlin, B.: Evaluation of bactericides by egg injection method with special reference to development of technic. Proc. Soc. Exptl. Biol. Med., 1942, **49:**27–31.
122. Woodruff, H. B., and McDaniel, L. E.: The antibiotic approach. Society for General Microbiology, Symposium No. 8, pp. 29–48. Cambridge University Press, 1958.
123. Woolley, D. W.: A Study of Antimetabolites. John Wiley and Sons, New York. 1952.
124. Woolley, D. W. The designing of antimetabolites. Society for General Microbiology, Symposium No. 8., pp. 139–162. Cambridge University Press, 1958.
125. Woolley, D. W.: Antimetabolites. Science, 1959, **129:**615–621.
126. Work, T. S., and Work, E.: The Basis of Chemotherapy. Oliver and Boyd, Edinburgh. 1948.
127. World Health Organization: Standardization of methods for conducting microbic sensitivity tests. Second report of the Expert Committee on Antibiotics. W.H.O. Technical Report Series, No. 210. Palais des Nations, Geneva. 1961.
128. Wyss, O.: Chemical disinfectants. Ann. Rev. Microbiol., 1948, **2:**413–434.
129. Yow, E. H.: A re-evaluation of sulfonamide therapy. Ann. Inst. Medicine (Chicago), 1955, **43:**323–332.

Chapter 7

MICROBIAL VARIATION AND GENETICS

Prior to the development of methods of isolation of bacteria in pure culture, *i.e.*, as clones, and the study of such isolates, these microorganisms were regarded as closely related or identical but of highly variable morphology. This view reached its zenith in 1877 with Nägeli who postulated but a single species to which all bacteria belonged. This early concept of extreme pleomorphism was almost, but not quite, shattered by the study of bacteria in pure culture and the consequent development of a strict monomorphism by Koch and others which admitted substantially no change in the characteristics of bacteria in pure culture. It subsequently became clear that a more realistic view is a compromise between these two extremes, *i.e.*, bacteria in pure culture have a continuing identity which is subject to variation within limits, although as yet these limits cannot be rigidly defined. Further, as the viruses have become better known, it has become evident that these forms too are subject to substantially the same kind of variability as the bacteria.

In a consideration of this aspect of microorganisms, it is essential to have clearly understood the reference point, or norm, from which deviations occur. Such a norm is difficult, if not impossible, to define in any absolute way. There is a tendency to assume as the "normal" the character of a microorganism occurring under conditions of man's devising such as the properties of a microorganism growing or grown under laboratory conditions. One of the most obvious examples of the potential fallacy in such an assumption is the varied morphology of the symbiotic nitrogen-fixing bacteria of the genus Rhizobium between the regular, rod-shaped form occurring on laboratory culture mediums and the so-called bizarre forms observed in its natural habitat, the nodules of leguminous plants, which is illustrated in Figure 50. In a very real sense there is no "normal," but rather many base lines which can be defined only in the context of the particular conditions under which the microorganisms have been grown and, to complicate matters further, often its past history. Any deviation, then, must be defined in terms of the reference point arbitrarily taken.

THE "UNIQUE" FEATURES OF MICROORGANISMS

In many respects microorganisms are seemingly unique among living things, and in a general sense this "uniqueness" is of three kinds. One is a consequence of their extremely small size which has been touched upon earlier in relation to the morphology and growth of these forms. The accompanying simplicity of structure necessitates the use of differen-

Figure 50. The morphology of Rhizobium. *Left,* a smear from a pure culture on laboratory mediums; note the regularity of the "normal" bacillary morphology. *Right,* smear from a clover root nodule showing swollen, branched and cococid forms, the so-called bacteroides. Fuchsin; × 1050.

tial criteria of a more subtle kind than those of comparative anatomy applicable to higher forms, *viz.,* physiological and immunological properties. It is obvious that when a microorganism is so characterized, variability consists of deviation from some base line in these respects. Because such criteria are not usually applicable to higher forms, the impression is produced that the microorganisms are somehow "different." This is clearly fallacious as indicated by studies on the physiological genetics of higher forms and by the parallelism between the immunological relation of, for example, the serum proteins of mammals and their phylogenetic relationships based upon a comparative anatomy (Chap. 13).

The microorganisms share with other cells the essentially abstract environment in which factors such as exaggerated forces of surface tension and the predominance of interfacial phenomena at near-molecular or macromolecular dimensions play a large part. They differ from the differentiated cells of higher forms, not only in being smaller, but more significantly in being independent, complete units. As such they are not protected from marked variation in the immediate environment by the other components of a multicellular structure or by extensions of the structure such as regulatory mechanisms of homeostasis present, for example, in the mammal. Thus the directive mechanisms that maintain the integrity of the microorganism are not far removed from a variable external environment, and it might be anticipated that their functioning could be altered by extracellular influences. Such differences between microorganisms and other living things are, of course, a matter of degree rather than kind.

A second kind of seeming uniqueness derives from the extremely rapid rate of reproduction of the microorganisms. Whether it is a matter of environmental selection of the fittest from a mass of pre-existing heterogeneous individuals or of mutants conforming more closely to the requirements of a particular environment, variant forms appear relatively rapidly. This might be taken to suggest that the microorganisms are unusually facile in this respect or, from another point of view, are relatively unstable with respect to at least certain of their characteristics. When the period of 30 years generally taken to represent a human generation is contrasted with that of 20 minutes for a rapidly growing microorganism, it becomes clear that the ready adaptability of microorganisms may be accounted for in large part by rates of reproduction. For example, mutation rates of bacteria are of the same general order as those demonstrable in

more complex forms such as Drosophila, even though they appear much more rapidly in terms of elapsed time.

Finally, in contrast to many higher forms, the individual microorganism is not characterized except in a limited way, as with respect to size and shape of the individual cell. The statement that Bacillus X ferments such-and-such a sugar is not literally true. More accurately, it should be said that the sugar is fermented in the presence of a mass of many millions or hundreds of millions of the microorganism. This is not quibbling, but is rather, a recognition of the limitations imposed by the practical necessity for studying microorganisms as large populations, and recognition of this fact is of primary importance to an understanding of the mechanisms operative in microbial variation.

The properties of microorganisms must, then, be studied in terms of population biology. In the population, the individuality of its members is merged and disappears, and the characteristics of a population, expressed as statistics, are both more and less than the total character of the discrete entities that make it up. This is often a difficult matter to appreciate, for man reacts as an individual and conceives of the population to which he belongs as largely a matter of his own relation to it. Tippett has suggested that arriving at a concept of a population as an entity in itself is analogous to the appreciation of a fugue with its contrapuntal pattern as contrasted with the ease of following a simple harmony or tune.

For present purposes it is significant that variability is an inherent characteristic of populations; in fact, if each of its component entities were identical with all the others, it would lack such distinguishing characteristics as, for example, mean or median size, etc. So far as is known, living organisms are seldom if ever precisely identical, and a microbial population, such as a bacterial culture, is no exception but is seemingly homogeneous because it is studied as a population and individual differences are averaged out. Microbial variation is, then, a change in population character from which one infers, more or less successfully, causality on an individual basis.

It will be clear from the foregoing that microorganisms are not unique in the sense of being fundamentally different from other living forms. One may expect, therefore, to find that microbial variability has the same bases as variability of higher forms, although in some cases its expression may appear to differ. This has not only been found to be true, but, as will appear, study of variation in microorganisms has both broadened and pinpointed genetic mechanisms to an extent not possible in the study of higher organisms.

Here variation will be considered under two general heads, the kinds of variation observed, and the mechanisms operative in such variation.

OBSERVED VARIATIONS OF MICROORGANISMS

Microorganisms have been found to vary, sometimes widely, in all the characteristics made use of in their differentiation and identification. These include morphology, both macroscopic and microscopic; physiological properties, including ability to produce disease; and immunological character. Changes may appear suddenly, *i.e.*, in a single subculture, or they may become apparent only gradually, as in aged cultures or over many transplants or animal passages and some hundreds of generations. The rapidity of appearance, however, cannot be taken to have fundamental significance and, in fact, in many instances is subject to experimental manipulation. Variants may appear apparently spontaneously or may be seemingly induced as by making the culture

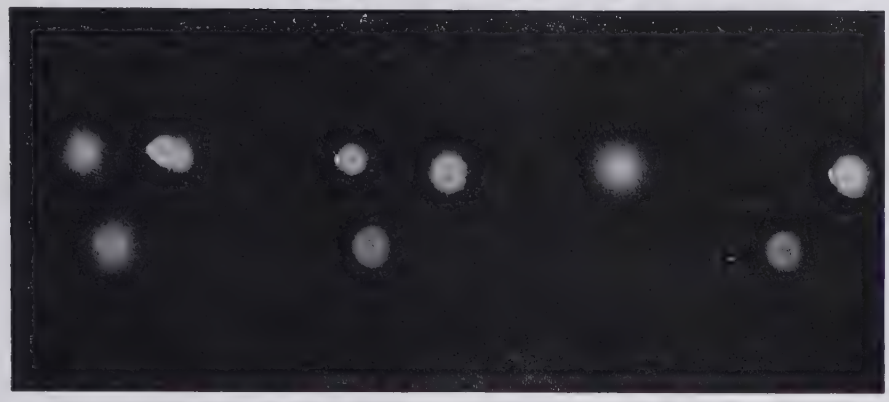


Figure 51. Smooth and rough colonies of *Salmonella typhi;* × 3. Culture on nutrient agar inoculated from an old broth culture.

medium mildly and specifically toxic by the inclusion of lithium chloride, antiserum, antibacterial substances, and the like, or by making it selective so that the expected variant is given enhanced survival value as, for example, by including a sugar in the medium in attempts to isolate fermenting variants of a nonfermenting parent strain. In fact, in practice the single most potent factor in the demonstration of microbial variants is the selective nature of the environment, which may be either inadvertent or planned. Without this factor the relatively rare variant or mutant cannot achieve the numerical status necessary to give an observable change in the population of microorganisms.

In any case, the variation is observed as a population characteristic in that the variant character of the individual cell is found in subculture, and the inference regarding the individual cell rests upon the assumption that the variant character breeds true for at least this long.

MORPHOLOGICAL VARIATION

Variation in the morphology of bacteria may be considered under two general heads: colonial morphology or that of masses of cells, and the morphology of individual bacterial cells.

Bacterial Dissociation. A remarkable type of bacterial variation, whose most obvious outward manifestation is a change in the type of colony formed on semisolid mediums, was observed by Baerthlein in Germany (1918), Arkwright in England (1921), and de Kruif in the United States (1921). The phenomenon was termed by de Kruif bacterial or microbic dissociation, a term which has, in spite of certain undesirable features, been generally adopted.

Colonial morphology. The ordinary laboratory culture, particularly old broth cultures, when plated out on an agar medium develops into two kinds of colonies; one, smooth (S), round, convex, and shining; the other, rough (R), irregular, flattened, and wrinkled. In addition to these obviously different extreme colony types, all degrees of intermediate (SR and RS) types may usually be found. The transformation of the S form, usually regarded as the "normal," into the R type is, as indicated by the presence of intermediate types, probably a gradual process. The smooth form of the great majority of bacteria, while breeding true for a number of test tube generations, shows a constant tendency to change to the rough type; one of the consequences of this tendency is that most stock laboratory cultures contain a large proportion of roughs, and sometimes such cultures are completely lacking in smooth forms.

A third kind of colony, the M or mucoid, is also commonly recognized, characterized by a slimy, mucus-like consistency and appearance. The mucoid character may be associated with the

S or R colonial types to give mixed or intermediate forms, but it is thought by many that S is intermediate between M and R and that the succession is $M \rightleftharpoons S \rightleftharpoons R$. In individual cases other colonial forms may be observed; the hemolytic streptococci, for example, occur in a form designated "matt" by Todd and the British workers which has a ground-glass appearance when viewed by reflected light. Still another form, the dwarf colony or D type, may be produced by experimental manipulation and is sometimes isolated from the natural habitat of the microorganisms. Colonial differences, often very slight in character but frequently associated with virulence and other physiological properties, are apparent when the culture is examined under low magnification by oblique, transmitted light as described by Henry.[65]

The transformation of S to R, though occurring in ordinary cultures, may be hastened by a number of procedures, such as the inclusion of lithium chloride or anti-S immune serum in the medium in which the organisms are cultured. Smooth cultures lysed by bacteriophage give rise when incubated to a secondary growth which often consists entirely of R forms. The change from R to S is considerably more difficult, and the rough forms show little tendency to revert under ordinary cultural conditions. The reversal may, however, be accomplished in most cases, one of the most effective methods being the cultivation of the R form in the presence of anti-R immune serum. Reversion of the R type may also be brought about by animal passage.

Properties of S and R variants. The bacteria which make up the smooth and rough colonies differ from one another in a number of characteristics, some of which are usually regarded as of deep-seated biological significance.

The distinctions between the S and R types given in the accompanying tabular form represent a generalization to which there are a number of exceptions. For example, what is usually regarded as the "normal" form of the anthrax bacillus is the rough form, and in this case the organism is virulent in the rough form but avirulent in the smooth form. Similarly, the "normal" virulent form of the hemolytic streptococci is finely granular rather than smooth, and the avirulent form which arises from it has lost its type-specific antigen but is smoother than the virulent form. In spite of these and other exceptions, the differences noted above hold true in general.

Cellular morphology. Closer inquiry into the differences between S and R bacterial types indicates that the relation between colony form and certain other characters is not in the nature of a casual correlation but rather the result of an intimate association. The association between capsules and virulence has been pointed out previously, as has been the role of the capsule in giving the colony of encapsulated bacteria a slimy, viscous consistency. It is not unreasonable to expect, then, that a bacterium which lacks a capsule is also avirulent, or relatively so, and that its colonies will be dry and possibly wrinkled in appearance. The loss of a capsule likewise alters the nature of the bacterial surface; the absence of carbohydrate material allows the lipids present in the cell membrane to determine its character; in consequence, it becomes hydrophobic rather than hydrophilic, and the organisms tend

Contrasting Properties of S and R Varieties

S TYPE	R TYPE
colony usually smooth and viscous	colony often dry and granular and may be larger
pathogenic forms usually virulent	pathogenic forms often relatively avirulent
form stable suspensions in salt solution	spontaneously aggregates and settles out in granular deposit from suspension in salt solution
encapsulated forms have capsules	capsules are diminished or absent
antigenically "complete"	often show loss of antigens, sometimes with the appearance of "new" antigens

to clump and settle out of salt solution or the nutrient broth of a culture. The granular consistency of many of the rough colony types is accounted for, in part, by postfission movements of the cells. The smooth forms of some of the intestinal bacilli separate after fission and slip to lie side by side with one another. The cells of the rough forms, on the other hand, tend to remain attached after fission with the formation of chains which bend at sharp angles here and there. The development of bizarre shapes by some of the rough forms may possibly be a result of the absence of capsular material and change in the nature of the cell surface. The nature of the association between roughness and reduced biochemical activity is unknown.

Induction of dissociation. Experimentally, the inclusion of antiserum to the S or R form will frequently result in the appearance of the other form; the occurrence of avirulent R forms of pathogenic microorganisms isolated from chronic carriers of the infection is presumed to be a consequence of the effect of antibody developed to the virulent S form. Most often the change, especially the S → R, occurs in old liquid cultures and is an effect of accumulation of metabolites. The role of metabolites in the S → R dissociation has been strikingly illustrated by the studies of Braun and his colleagues[33, 56] on Brucella. Smooth strains cultured in synthetic medium containing asparagin as the sole source of nitrogen multiply in the usual way, but after some days' incubation are replaced by R forms. The occurrence of R forms coincides with the accumulation of *d*-alanine which is specifically toxic to the S but not the R form. The S → R shift is prevented by including sodium pyrophosphate in the medium. This substance forms complexes with manganese and magnesium, trace elements that act as catalysts in alanine formation; the effect of pyrophosphate is in turn antagonized by the addition of manganese or magnesium. Smooth virulent forms, differing from the original smooth type in that they are resistant to alanine, may also be isolated. It is of considerable significance that the effects of amino acids may occur *in vivo* also. For example, in the case of *Salmonella typhimurium*, virulence for the mouse was greatly enhanced by administration of threonine to the animals; a more highly virulent, threonine-resistant variant, occurring as less than 1 per cent of the original inoculum, was selected *in vivo*.[102]

Variation in Cell Structures. As indicated above, colonial morphology is in part a function of the morphology of the individual cells making up the colony, and one of the most important factors in the differential morphology of the S and R variants is the occurrence of hydrophilic polysaccharide on the surface of the cell. As pointed out elsewhere, this frequently takes the form of a layer or morphologically demonstrable capsule on the outer surface of the cell.

Capsules. Capsule formation or, from the physiological point of view, the synthesis of capsular substance usually of polysaccharide but sometimes of polypeptide nature, is very common among the bacteria, but a morphologically demonstrable capsule is not always apparent. Capsule formation is dependent in large part on the environment, and the most favorable medium in the case of pathogenic bacteria in which capsule formation is associated with virulence is the body of the susceptible animal. The anthrax bacillus forms a heavy capsule in the infected animal, but there is little or no evidence of a capsule when the bacilli are cultivated on laboratory mediums. The pneumococcus, likewise heavily encapsulated in the infected animal, also forms a capsule in artificial mediums, but the capsule is best developed when the medium is enriched with blood or serum and carbohydrate. Among the pathogenic forms, capsule formation tends to diminish with continued cultivation on artificial mediums, probably because these furnish a somewhat less than optimal environment, and is restored by animal passage of the bacteria.

The heavily encapsulated saprophytic

bacteria, such as the dextran- and levulan-forming cocci of the genus Leuconostoc, show no tendency to loss of capsule formation in culture provided that carbohydrate is supplied, and it seems probable that the artificial medium is more nearly optimal than it is in the case of the more fastidious pathogenic bacteria. In general, then, capsule formation is determined in large part by the environment, may be a quantitative matter, and is reversible.

Capsule formation may be specifically inhibited, however, the most effective means being the inclusion of antiserum to the capsular material in the culture medium. The loss of capsule formation under such circumstances, or when it is relatively complete by thorough prolonged culture on artificial mediums, results in the S–R dissociation, and the lack of a capsule is characteristic of the rough variant. As indicated above, this loss is also reversible, though frequently only with considerable difficulty. Whether it is to be regarded as basically different from a gradual diminution in the amounts of capsular substance synthesized through continued cultivation in a less than optimal environment is not clear.

Flagella. The occurrence of flagella on motile bacteria is also subject to variation. There appears to be little or no variation with respect to numbers or position of flagella, but their presence or absence is variable. Nonmotile variants of motile bacteria are occasionally, though not commonly, observed, and these appear to be stable forms with little tendency to reversion to the motile form. In the case of very actively motile bacteria such as Proteus, colonial morphology is affected by motility in that the motile bacteria "swarm" and the growth spreads in a thin film over the surface of an agar medium, while the nonmotile variants form discrete colonies.

The formation of flagella is subject to environmental influence also. Actively motile species kept in stock culture on laboratory mediums tend to lose their motility, and potentially motile bacteria may be motile when cultivated at one temperature but not at another. The inclusion of antibacterial substances such as phenol in the medium in toxic but nonlethal concentrations inhibits the formation of flagella by bacilli of the Salmonella group; in fact, one method of obtaining these forms free from flagella and the antigenic substances associated with them is culture on nutrient agar containing 0.1 per cent phenol.

Spores. The spore is a consequence of the aggregation of cell substance within a spore case during spore formation. It is this process rather than the formation of a structure of the vegetative cell that is subject to variation. Like capsule formation, it is essentially a physiological process but with morphological consequences. Spore formation is affected by environmental factors, and asporogenous variants which breed true also occur. In the case of the anthrax bacillus, for example, spores are formed only when the bacteria have access to free oxygen; only vegetative forms are found in the infected animal until the bacilli are exposed to air by tissue decomposition or autopsy and then spore formation occurs. Similarly, spores are formed most rapidly at 32° C., less so at 37° C., and not at all when the culture is incubated at 42° C. The inhibition of spore formation by the anthrax bacillus by high temperatures is a temporary one in that when the temperature of incubation is reduced spore formation again occurs, but if cultures are maintained at 42° C. for many transfers the ability to form spores is apparently permanently lost. In general, however, spore formation is a relatively stable character.

L forms.[38, 77, 78, 79] A kind of variation known as L variation was first observed by Kleineberger in cultures of *Streptobacillus moniliformis* (one of the causative agents of rat-bite fever) and subsequently by other workers, especially Dienes, in cultures of a variety of bacteria. The bacteria become more transparent and stain with difficulty or form granules which unite and grow into the amorphous bodies of variable size known as L bodies. These bodies may multiply or give rise to bacterial

cells morphologically indistinguishable from the parent strain.

The significance of these variants is uncertain. In morphology they are similar to the pleuropneumonia microorganisms (Chap. 27) and appear to be etiologically related to polyarthritis of rats. Whether or not the L bodies are variants of common bacteria and have more than a superficial relation to the pleuropneumonia organisms is uncertain. It is also possible that these bodies represent naturally occurring protoplasts of greater stability than those prepared by enzymatic digestion of the cell wall (Chap. 3); if so, they could be regarded as bacterial variants which are unable to form a complete cell wall structure.[88, 132, 134]

Aberrant Forms. The occurrence of aberrant or "abnormal" forms of bacteria, differing from the presumably normal morphology of recent isolates and cultures on favorable mediums, is commonly observed. In the earlier literature these are called involution forms and regarded as primarily degenerative in character. While this term is applicable in some cases, it is not necessarily appropriate in all, for some kinds of bacteria show varied morphology as a rule rather than an exception.

The tendency to varied morphology is in part an effect of the environment on the cell and in part a function of the relative rigidity of the cell structure. For example, a transitory alteration in cell shape may be produced by culture of bacteria in toxic, but not lethal, concentrations of substances such as NaCl or certain antibiotics. The distortions produced in the first instance are probably in large part an osmotic effect, and in the second seem to be a consequence of inhibition of processes of cell division while synthesis of intracellular substances continues, giving rise to swollen forms. Or, the inclusion of surface tension depressants in the culture medium frequently results in the growth of bacilli in the form of long filaments and similar phenomena. Such aberrant forms are usually viable and on subculture produce normal progeny.

Bizarre forms are also commonly observed in old cultures, particularly in liquid mediums. This is probably a consequence of the presence of relatively large numbers of dead and dying cells and possibly also of the toxic effects of accumulated products of metabolism. When the bacterial cell is dead or dying, its structure begins to break down with autolytic changes. The protoplasm becomes granular and may escape into the surrounding medium, coccoid forms of bacilli and other swollen forms result from the breakdown of the osmotic barrier, etc. For example, the pneumococcus produces peroxide as a ter-

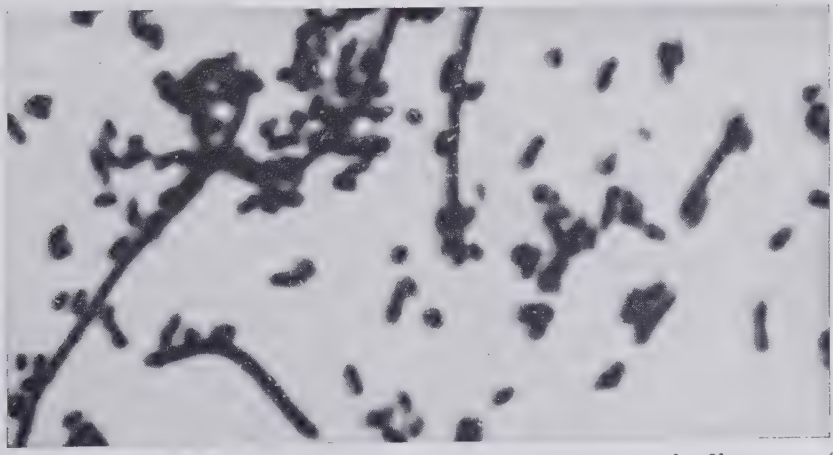


Figure 52. Involution forms of the typhoid bacillus; fuchsin stain. Note the filamentous forms and elongated rods mixed with the typical forms. What appear to be buds on the filament are probably adjacent cells. The poor resolution at these high magnifications is apparent. × 3500.

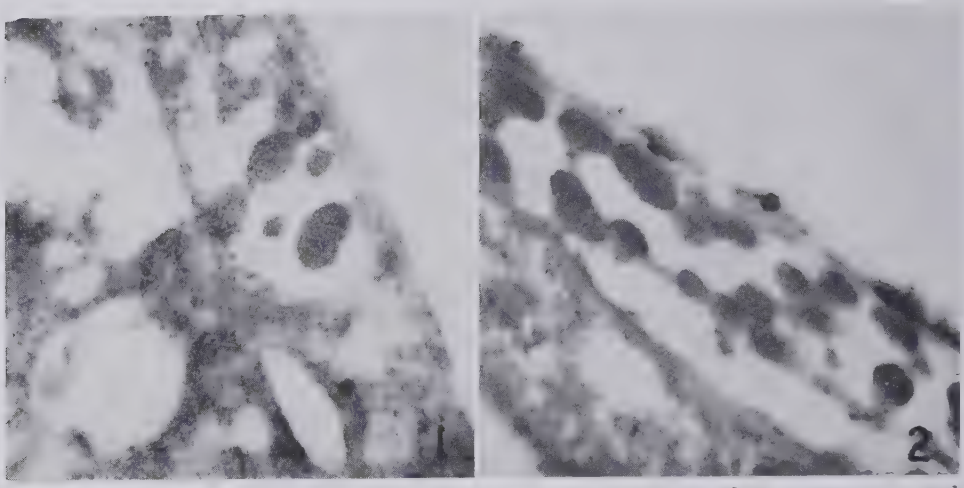


Figure 53. The varying morphological types of feline pneumonitis virus during one-step growth in chorioallantoic ectoderm. *Left,* the ovoid granulated forms occurring at 15 hours, at point *1* shown on the growth curve in Figure 54. *Right,* the smaller forms at 20 hours, at point 2 on the growth curve. Electron micrographs, × 15,000. (Litwin.)

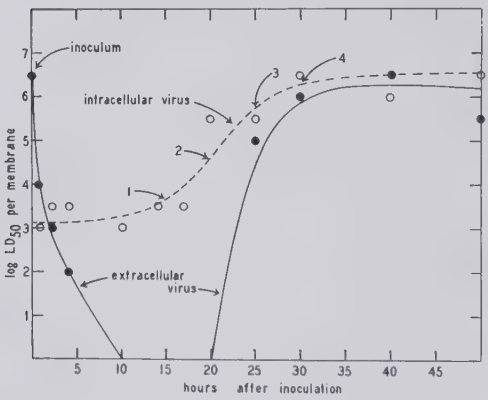


Figure 54. One-cycle growth curve of feline pneumonitis virus in chorioallantoic ectoderm. The dotted line represents intracellular virus, and the solid line that occurring free. The points labeled *1, 2, 3,* and *4* on the intracellular virus growth curve are the stages in the cyclic development of the virus illustrated in Figures 53 and 55. (Litwin.)

minal product of respiration, but no catalase, so that peroxide accumulates to lethal concentrations, killing the cells. These bacteria contain intracellular proteases, and autolytic changes set in rapidly. One of the first indications of this is a loss of the ability to retain the Gram stain, and even in 24-hour cultures many cells are found to be gram-negative. Similarly, the cholera vibrio is a fragile cell and may be broken up mechanically more readily than most other bacteria; it also contains a powerful intracellular protease; and it is not surprising that it is notorious for its morphological variability. Such cells are often dead in that they do not reproduce on subculture and may be regarded as involution forms.

Still other bacteria, notably forms such as Bacteroides and the pleuropneumonia organisms referred to above, seem to be so fragile in structure that they do not occur as a single or predominant morphological type. The Bacteroides are rod-shaped but occur in filamentous and other varied forms, while the pleuropneumonia organisms have a widely varied morphology, including coccoid forms, symplastic masses, and ring forms. Such varied morphology under standard conditions of culture is, however, the exception, and the great majority of true bacteria, or Eubacteriales, are remarkably constant in this respect.

Microbial Life Cycles. The occurrence of morphological variation among the bacteria inevitably raises the question of whether morphological variants are random events or have a deeper significance as expressions of cyclical events in the life history of the microorganism.

There is no doubt that simple sequen-

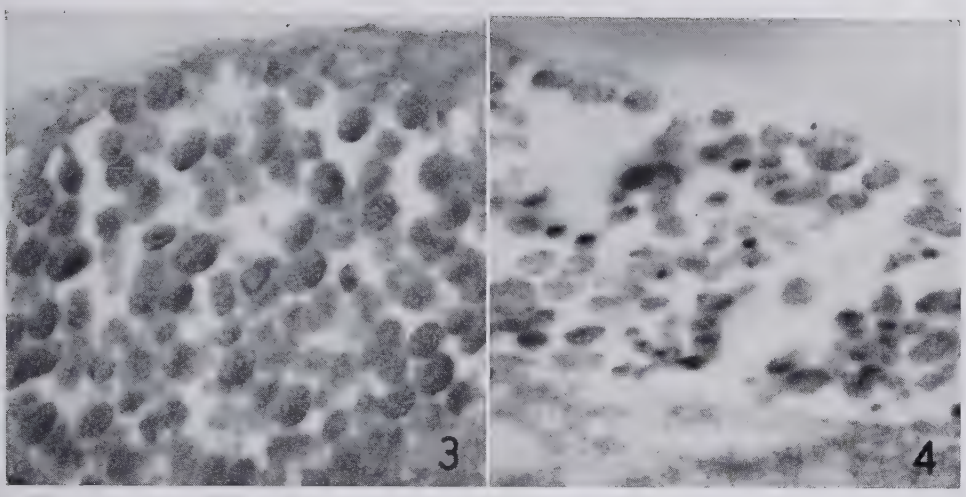


Figure 55. The varying morphological types of feline pneumonitis virus during one-step growth in chorioallantoic ectoderm. *Left,* the smaller forms have increased in numbers, corresponding to point 3 on the growth curve shown in Figure 54. *Right,* mature forms occurring at the end of the growth cycle at 30 hours and showing two morphological types, the smaller dense-centered bodies and the larger granulated forms. Electron micrographs, × 15,000. (Litwin.)

tial developmental changes expressed morphologically occur. Obvious examples are the spore-vegetative cell sequence occurring during the growth of spore-forming bacteria, the sequence of morphological types occurring during the growth of a culture and designated cytomorphosis, and the successive zoogleal and monad or swarmer stages of Nitrobacter. Such sequential changes can hardly be taken as expressions of life cycles such as, for example, that of Plasmodium.

The evidence for the occurrence of bacterial cyclogenies is substantially a matter of the observation of cells of varied morphology, in old cultures or similar unfavorable circumstances, and their arrangement in an order that may be taken to represent a sequential relation. The relatively constant morphology of most kinds of bacteria under defined conditions of culture thus becomes but one stage in the life cycle of the bacterium which is maintained to the exclusion of other stages by the environment. The fallacy lies in the arrangement of morphological variants, for it is necessarily subjective rather than objective, and the evidence is less than unequivocal. The possibility of the occurrence of successive developmental stages is not, of course, eliminated, but in any consideration of this kind it must be kept in mind that the organization of the microbial cell is largely on, at most, a macromolecular level, and variation need not have morphological expression. In fact, the great bulk of variations are physiological (see below) and are only rarely associated with morphological changes.

Virus replication cycles. As indicated elsewhere (Chap. 4), replication of the viruses and virus-like agents is characterized by the occurrence in time sequence of successive, differentiable stages. For the most part these stages are separable by immunological and infectivity criteria, and, with rare exceptions, notably the protein head structure of incomplete coliphage, these stages have no morphological counterparts.

Among certain of the larger viruses, however, varied morphology of the virus particle may be observed. The differentiation of the intracellular matrix or inclusion body into particles of pox virus, heterogeneous with respect to the amount and distribution of electron-dense, presumably nucleic acid, material within the particle has been described elsewhere (Chap. 4). These forms occur

admixed within the host cell and, while they may be arranged in an order, the arrangement is as yet arbitrary rather than determined by time or other objective factor.

Members of the psittacosis-lymphogranuloma group of viruses have been studied in most detail in this respect for it was apparent quite early that morphologically different virus particles occur during the course of the reproductive cycle. Their occurrence in an orderly sequence was suggested by studies with the light microscope,[135] and subsequently they were more precisely defined by electron micrography.[92] The growth cycle of feline pneumonitis virus in ectodermal cells of the chorioallantois of the embryonated hen's egg is completed in about 30 hours. By 15 hours the spherical elementary body, about 200 mμ in diameter, has increased in size to 500–700 mμ and contains an increased amount of electron-dense material, and at this point the growth cycle corresponds to the lag phase of bacterial growth. At 20 hours the bodies are somewhat smaller, 500 mμ, somewhat increased in numbers, and the vesicle within which they are contained in the host cell is larger. By 25 hours growth is exponential, the bodies are transitional in size and ranging from 200 to 500 mμ, and the vesicle practically fills the host cell. The end of the cycle is reached by 30 hours, with the appearance of two kinds of mature virus particles, the one a small spherical body about 200 mμ in diameter and containing a central body of electron-dense material, and the other a larger ovoid particle, 300 $\times$ 500 mμ, which is reticulate or granular in structure. The analogy to the successive stages of bacterial cytomorphosis during growth is striking, but, since the viruses of this group are set somewhat apart from the other viruses it does not follow that virus growth cycles in general might be expected to be reflected in such a succession of morphological types.

In summary, there is as yet no convincing evidence of the occurrence of bacterial life cycles other than the cytomorphosis occurring during the population growth cycle. The virus-like agents of the psittacosis-lymphogranuloma group show a similar pattern of successive morphological types of virus particle, but the clearly apparent stages in the developmental cycle of other viruses are characterized by immunological methods and infectivity.

PHYSIOLOGICAL VARIATION

Variations in the so-called physiological characteristics of a microorganism are not fundamentally different from morphological variations in that the latter involve the synthesis of cell structures, but in practice they are taken to be those which do not necessarily have a morphological expression. These include the ability of a microorganism to decompose sugars and other substrates with the appearance of characteristic end products (the biochemical properties of microorganisms), their nutritive requirements, susceptibility to infection with bacterial viruses, immunological character, and, in the case of pathogenic forms, virulence.

All of these may be equated on the common basis of the synthetic abilities of the microorganism. The decomposition of various substrates is obviously dependent upon the elaboration of the appropriate enzymes, and nutritive requirements are an expression of synthetic abilities in that a substance required for growth, in the sense that it must be supplied preformed, is one that the microorganism cannot synthesize. Susceptibility to infection with bacterial viruses is dependent, in part at least, on the presence of appropriate receptors in the cell wall structure, a product of synthesis. Similarly, immunological character is an expression of the antigenic specificity of components of the cell substance of the microorganism, or the products of its metabolism, both the result of synthesis. The nature of the virulence of pathogenic microorganisms is not fully understood, but it is indicated that the ability to produce disease

is in large part a consequence of the production of toxic substances, ranging from potent exotoxins to enzymatic or kinase-like activities that facilitate invasion of the tissues of the host (Chap. 9). Such substances are also products of microbial synthesis.

The identity of a microorganism in all these respects is thus referable to the operation of directed control mechanisms within whatever limitations are imposed by the environment. It follows that microbial variation arises from alterations in genetic control mechanisms and/or their functioning within the microcosm of the environment to give phenotypic expression of genotypic character.

Environmental Selection

The demonstration of microbial variants almost always requires the utilization of a selective environment such as a culture medium that may be differential as well as selective or an experimental animal or its equivalent in the case of virulence studies. This is because ordinarily only a minute portion of the microbial population differs from the parent strain, and its emergence from the population to recognizable proportions is usually dependent upon an environment which is more favorable to the development of the variant than of the parent strain.

This approach is applicable directly in the case of variants in which the change is positive, in the sense of the acquisition of the ability to ferment a sugar, to produce infection in an experimental animal or the development of resistance to an antimicrobial substance. For example, when a substrate which the parent strain is unable to utilize is present in a culture medium, the variant which is able to decompose it may be able to outgrow the parent strain. If the substance is a sugar it may be incorporated in an agar medium together with an indicator, such as an acid-base indicator or Schiff's reagent, so that the colonies of the variant will be colored while those of the parent strain are not. Or, when the parent strain requires, for example, an amino acid for growth, variants which are able to synthesize the amino acid grow in a medium lacking it while the parent strain does not. In the same way, a culture medium may include an antimicrobial substance, such as an antibiotic, which prevents the growth of the parent strain but not that of a resistant variant, or after inoculation with bacteria the medium may be inoculated with bacterial virus which inhibits a susceptible parent strain but not a resistant variant. Similarly, the virulent pathogenic microorganism grows readily in the tissues of the host, while the avirulent form fails to establish an infection and is destroyed by host defense mechanisms.

Variations involving losses require a different approach in that the growth of the parent is not suppressed in the absence of the substrate while that of the variant is. Consider a parent strain which does not require tryptophan and a variant which does; both will grow on a medium containing the amino acid, but only the parent strain on a medium lacking it. Individual colonies may be subcultured from the complete medium and tested, but this procedure is laborious and, for the isolation of such variants on any scale, an indirect method is required.

In this connection advantage may be taken of the fact that penicillin affects actively multiplying bacteria to a much greater extent than those which are not multiplying. An agar medium deficient in some nutrient, such as an amino acid, is inoculated and incubated for sufficient time to allow active multiplication of those cells which do not require the amino acid. It is then treated with penicillin to selectively destroy the actively multiplying cells, the activity of the penicillin neutralized, and the inoculated medium overlaid with agar medium containing the amino acid, or other substance, not present in the first medium. The cells which were unable to multiply in the absence of the substance in the first instance, and so were not affected by the penicillin treatment, grow out in the second medium.

Adaptation[35, 128]

For present purposes the term adaptation may be interpreted broadly as covering all microbial variations which are consistent with an increased physiological compliance with the demands of the selective environment. The adaptive response is of two kinds. One of these is actually a response to the environment in that it is induced, more or less directly, by an environmental factor. The other is not a response in the literal sense, and its adaptive character is a consequence of the appearance of a random mutant which, purely by chance, has greater survival value than the parent from which it was derived. In a general way, the induced variation usually develops slowly and the adaptation seems to be continuous, while spontaneous mutants appear abruptly and the adaptation which is dependent upon a series of mutations occurs as discrete steps. It is often not possible to distinguish between these two kinds of adaptation, and both may be involved in what seems to be a single adaptive process.

Attenuation

The term attenuation is ordinarily loosely used to mean altered virulence of a pathogenic microorganism. It may indicate a simple loss of ability to produce disease in a general sense or a reduction in virulence for one host species accompanied by an increase in virulence for another host species.

Loss of Virulence. In general, most pathogenic bacteria tend to lose virulence when kept in culture on artificial mediums. For example, pneumococci rapidly lose virulence for the mouse and in even a very few transfers on enriched mediums the minimal lethal dose increases by several hundredfold. Staphylococci, however, retain their virulence over many transplants on artificial mediums but eventually become relatively avirulent. A few bacteria, such as the anthrax bacillus, seem to retain virulence almost indefinitely, but this is not common. Such losses in virulence are general in character in that the bacteria become less virulent for a variety of experimental animals and presumably arise as a consequence of adaptation to an environment other than that of the animal body.

The artificial environment may be deliberately made somewhat toxic in order to bring about such changes. Simple aging in the case of Pasteur's cultures of the fowl cholera organism was sufficient to reduce virulence so that a fatal infection was not produced, and the attenuated strain of bovine tubercle bacillus known as BCG was carried on a bile-containing medium until its virulence was apparently completely lost.

Loss of virulence may be accompanied by changes in other characteristics such as marked diminution in capsule formation by the pneumococcus, a loss of golden pigment by *Staphylococcus aureus* and somewhat less fastidious nutritive requirements. In many instances the change is dissociative in nature, and loss in virulence is associated with a shift from the smooth form. In most bacteria, of course, virulence is markedly reduced by the S–R dissociation, and a change of this kind may occur *in vivo*, presumably under the influence of antibody; for example, the typhoid bacilli excreted by chronic carriers are often rough, avirulent forms.

Animal Passage. Virulence may often be restored by animal passage, *e.g.*, successive and repeated infection and reisolation of the bacterium through a series of experimental animals. It cannot be concluded that the microorganisms are individually altered in the restoration of virulence by animal passage, and it is more probable that the animal acts as a highly selective screen, separating out those few bacteria in the inoculum which multiply most rapidly in the tissues. The restoration of virulence is usually accompanied by a restoration of other characters associated with it such as capsule formation.

Modification of Virulence. The virulence of a microorganism may be modi-

fied by passage through another host species, and sometimes, though not invariably, virulence for the original host species is reduced. The classic example of attenuation by animal passage is that of Pasteur's attenuation of the rabies virus by adaptation to the rabbit brain. The original strain from dogs, "street virus," kills rabbits in about two weeks following subdural inoculation, after some twenty passages it kills in eight days, and after an additional twenty or more passages the period may be reduced to seven days but cannot be reduced further. This "fixed virus" will not produce rabies on subcutaneous inoculation and so may be used as an immunizing agent. Such attenuation or adaptation to a new host is very common among the viruses and includes the adaptation of a variety of viruses to the chick embryo, the adaptation of yellow fever virus to the mouse brain, etc. For example, on primary isolation in the embryonated hen's egg, influenza virus forms relatively little hemagglutinin for the chicken erythrocyte, but after a few passages hemagglutinin is formed in abundance. This has been designated the O-D variation of influenza virus and represents an adaptation to the new host tissue. This virus can be further modified to, for instance, the mouse lung, or its naturally occurring pneumotropism altered by successive passage in the mouse brain so that, like the adapted yellow fever virus, it becomes neurotropic.

Biochemical Variation

Variation in the biochemical properties of bacteria, such as fermentation of sugars, decomposition of proteins and amino acids, nutritional requirements, resistance to antibacterial agents, and the like, occurs with some frequency. Some of these develop more or less gradually on continued cultivation in selective mediums and resemble the process of attenuation of virulence.

Adaptation and Training. Such variation in nutritive requirements is the basis for the general observation that the more fastidious pathogenic bacteria are frequently more difficult to cultivate on primary isolation and enriched mediums are required, while, after being carried in culture for some time, they grow more rapidly and profusely and on somewhat simpler mediums. For example, on primary isolation *Brucella abortus* requires an increased carbon dioxide tension, but after a few subcultures this may be dispensed with. Similarly, the gonococcus and meningococcus require, on primary isolation, both an increased carbon dioxide tension and an enriched medium, usually a heated blood medium. After a few subcultures, however, it is no longer necessary to supply carbon dioxide, and eventually growth occurs in simpler mediums. The adaptive process may be initiated, or markedly accelerated, by culture of bacteria in mediums designed to select variants. Pathogenic staphylococci become less fastidious in their nutritive requirements when maintained in the laboratory as noted above, and variation in this direction may be accelerated by forcing the bacteria to grow in a medium that is made successively less and less complex. For example, the amount of a required amino acid present in a synthetic medium may be gradually reduced over successive transplants, until finally the microorganism no longer requires it. By such means fastidious staphylococci have been trained or adapted to grow in a simple medium containing only glucose and an ammonium salt. Similarly, typhoid bacillus strains requiring the single amino acid tryptophan may be trained to grow in its absence, and strains of dysentery bacilli capable of growing in a glucose-ammonium salt medium, supplemented only with nicotinic acid, may be adapted to dispense with the vitamin.

Ability to ferment carbohydrates may also be either acquired or markedly enhanced by continued cultivation in the presence of the substrate or by very heavy inoculation of highly specific mediums in which the substrate is the only nutritive material. Still other adaptations

such as to changes in pH, increased incubation temperature, and the like also occur but are usually not marked.

Discontinuous Variations. Some microbial variants appear much more rapidly than those developing in the kind of adaptations described above, often occurring in the first culture on a selective medium. They are regarded as expressions of a sudden, discontinuous change, or mutation in the genic sense, in the genetic apparatus of the microorganism.

Lactose fermentation. The classic example of this kind of variation is that shown by certain strains of coliform bacilli which are atypical in that they are unable to ferment lactose, but which throw off lactose-fermenting variants. This phenomenon was observed as early as 1907 by Massini, and the strains of coliform bacilli were named *Bacterium coli mutabile.*

The phenomenon is demonstrated by culture of the bacteria on an agar medium containing lactose together with an acid-base indicator which turns red in the presence of acid. The primary growth consists of white colonies of the non-lactose-fermenting parent strain, and on continued incubation a secondary growth of the lactose-fermenting variant appears in the form of red papillae on the white colonies. On subculture on the same medium, the lactose-fermenting variant breeds true, while the parent strain again produces white colonies on which red papillae appear. The enzyme produced by the variant, but not by the parent strain, is the adaptive enzyme β-galactosidase. As an adaptive enzyme (see below), it is produced only in the presence of an inducer, in this case lactose and certain related compounds, and the variant is characterized by the ability to form the enzyme and its potentiality is realized in the presence of the substrate. In genetic terminology, therefore, the genotype of the parent strain is lac– and that of the variant lac+, but both are phenotypically the same in the absence of inducer.

Other variations. Many other variants of this rapidly appearing kind are well known and have been studied extensively, especially variants resistant to chemotherapeutic agents (see below) and those requiring essential metabolites such as amino acids and purines. These distinctive characters have been used as markers in studies of the genetics of microorganisms for they behave in a manner consistent with the assumption that they are determined by genes or their equivalent.

Analogous variations occur among the viruses also. The characteristics subject to variation are not physiological, since these agents apparently have no independent metabolism, but are characteristics such as plaque size and appearance and burst size of the bacterial viruses, and immunological changes, virulence, and cytotropism of animal viruses, of which influenza is the best known in this respect.

Variation of this kind cannot be dissociated from the adaptive processes described above. Such a variant, with some small advantage in growth rate over the parent strain in a selective environment would displace the parent type more or less slowly to give the appearance of a slowly developing adaptive response. The microbial population tends to obscure the nature of the processes of variation, but it is likely that spontaneous mutation plays a significant part in adaptation.

Figure 56. Colonies of *E. coli mutabile* on lactose agar. Note the lactose-fermenting papillae of the variant appearing on the non-lactose-fermenting colonies (Parr).

Resistance to Chemotherapeutic Agents[1, 55, 107, 114]

The development of strains of microorganisms resistant to chemotherapeutic agents was observed as early as 1907 in connection with the parafuchsin therapy of trypanosome infections in mice. It has been observed since that microorganisms will become resistant to a variety of compounds, including aniline dyes (parafuchsin, acriflavine), aromatic arsenicals (Atoxyl, arsacetin, tryparsamide), aromatic compounds of antimony (stibenyl, Fuadin), suramin (Bayer 205), styryl quinoline derivatives, guanidine compounds, sulfonamides, and antibiotics.

Prior to the introduction of sulfonamides, therapy of infectious diseases was limited to the diseases caused by spirochetes and animal parasites. When sulfonamides, and within a few years antibiotics, became available, they became so widely used that the problems created by microbial resistance to chemotherapeutic agents assumed large proportions.

The facility with which resistant strains of microorganisms develop is a property of both the microorganism and the antimicrobial substance. For example, staphylococci become resistant to many kinds of antibiotics very readily, the gonococcus becomes resistant to sulfonamides but apparently less readily to antibiotics, while pneumococci and streptococci are only occasionally resistant to sulfonamides or antibiotics but not to a practically important degree. Conversely, it is characteristic of streptomycin that almost all kinds of bacteria become resistant to it, often very rapidly. Microbial resistance may be considered here from two points of view, that of natural occurrence and therefore inferentially *in vivo*, and that of experimental production in the laboratory or *in vitro*.

Resistance Acquired *in Vivo*. The development of resistance under natural conditions of use of antimicrobial substances for therapeutic purposes is expressed as an increased proportion of resistant strains among those found in disease conditions or in carriers. When the sulfonamides were first introduced into general use, they were effective in the treatment of the majority of cases of gonorrhea, but within a relatively short time, three or four years, only a minority of cases of this disease could be treated effectively with these compounds, and strains of gonococcus isolated from refractory cases were shown to be resistant. Subsequently the response to repository penicillin has become less satisfactory, and is considered to be a factor in the increased prevalence of gonorrhea.[24, 54] With the consequent decline in the therapeutic use of sulfonamides, there is now evidence that the prevalent strains are becoming more sensitive to sulfonamides, and these compounds may again be effective.[67, 136]

Similarly, the proportion of staphylococcus strains resistant, first to penicillin, subsequently to tetracyclines and other antibiotics, and often showing multiple resistance, increased at a rapid rate as the antibiotics became generally available and widely used.[8] There is evidence too that there is an appreciable sulfonamide resistance developing among the dysentery bacilli.[133]

The occurrence of resistance is related to the frequency with which antibiotics are used. Staphylococcal resistance has been studied most intensively, is of the most practical importance, and may be used here as an example. Resistance to penicillin, the first generally used antibiotic, appeared first, and then resistance to tetracyclines and chloramphenicol, and subsequently to newer antibiotics such as erythromycin. Chloramphenicol contains a nitro group (Chap. 6), and after a number of cases of aplastic anemia were associated with its continued administration, it was used much less commonly. Coincident with this, there was a decline in the proportion of staphylococcus strains resistant to it. This, and other evidence, such as that of reversal of sulfonamide-resistance among gonococci, suggests that, as measured by the proportion of resistant strains, bacterial resistance in nature is not established permanently, but rather is maintained by continued general use of a chemotherapeutic agent. The tend-

ency to reversion to sensitivity, coupled with the variety of chemotherapeutic agents now available, opens the way to circumventing the problems of effective chemotherapy. In Hammersmith Hospital in London, for example, it has been possible to reverse the tendency to increasing antibiotic resistance of staphylococci and reduce the frequency of staphylococcal infection by balancing the kind of chemotherapeutic agents used against the observed frequency of resistance, together with special attention to sterilization and prevention of cross-infection.[9]

While, as might be anticipated, resistant strains occur in greatest proportion in hospital populations, for the most part such resistant strains are not introduced by the patient, but rather infection of the patient with resistant strains is acquired from hospital personnel. The source of infection is most often carriers, usually nasal carriers, and the incidence of nasal carriers of resistant staphylococci among surgeons and surgical personnel may become high. It is not uncommon to find that a wound infection following surgery is one with staphylococci having multiple resistance which does not respond to treatment with the commonly used, and generally available, antibiotics. The problem so created has reached such proportions that bacteriological control of surgical personnel has become necessary in some instances. Such studies as have been made of the incidence of resistant staphylococci in the general population suggest a proportion of perhaps 20 per cent, though this may vary widely and be much lower. In contrast, the proportion of penicillin-resistant staphylococci in hospital personnel commonly ranges from 60 to 85 per cent in this country and in Western Europe. The general problem of the incidence and behavior of bacterial strains resistant to chemotherapeutic agents has been studied intensively.[44, 45, 46, 47, 75, 89]

Origin of resistant strains. It is not completely clear whether resistant strains of microorganisms are pre-existing and make up a small, possibly minute, portion of all strains, or whether they arise for the most part in the individual undergoing chemotherapy. In the first instance, when the microorganism is primarily a parasite of man, the widespread use of a chemotherapeutic agent alters its environment to favor the survival of resistant strains. Under such circumstances it is to be expected that the proportion of resistant strains would increase.

It is well established, on the other hand, that resistant strains of microorganisms appear in persons originally infected with sensitive strains who are undergoing chemotherapy, especially prolonged therapy. This is most commonly observed in tuberculosis in which the prolonged therapy required, combined with the marked tendency of the microorganism to develop resistance to the kinds of chemotherapeutic agents used—streptomycin, *p*-aminosalicylic acid (PAS), and isoniazid—facilitates the emergence of resistant strains of the tubercle bacillus. When such infections are treated with a combination of two chemotherapeutic agents, the occurrence of resistant strains is markedly reduced. Many detailed studies have been made, and in a representative investigation[109] it was found that when patients were treated with isoniazid alone, 6 per cent yielded isoniazid-resistant strains of tubercle bacilli at the end of one month, and 58 per cent at the end of six months. When the disease was treated with combined antimicrobial substances, streptomycin in combination with PAS or with isoniazid, at the end of three months only 2 per cent of patients yielded streptomycin-resistant strains in the first instance and isoniazid-resistant strains in the second. Still other patients treated with PAS only prior to the institution of combined therapy with streptomycin and PAS showed streptomycin-resistant strains in 70 per cent at the end of three months of combined therapy when their tubercle bacilli had been initially resistant to PAS, but this was found in only 9 per cent of those whose bacilli were initially sensitive to PAS.

The suppressive effect of combined

therapy on the emergence of resistant strains supports the assumption that the resistant strain is made up of the progeny of a resistant mutant, and the growth of a mutant resistant to one of two chemotherapeutic agents used in combination is suppressed by the other. Assuming that the appearance of such mutants is a random, and therefore independent, event, the probability of occurrence of a double mutation is given by the product of the probabilities of each and becomes extremely small.

If the spontaneous mutant is the origin of resistant strains of microorganisms, it is immaterial whether these emerge in the general untreated population or in the infected individual undergoing chemotherapy. In either case the chemotherapeutic agent provides the selective aspect of the environment, either generally or in the treated individual. If, however, resistance develops only in the presence of the antimicrobial agent, suggesting that its emergence is specifically induced, the treated individual is the source of such strains.

Induced Resistance. Almost as soon as the antibacterial chemotherapeutic agents became available it was found that strains of microorganisms resistant to them could be readily produced in the laboratory. The usual procedure is to grow the bacteria *in vitro* or *in vivo* in the presence of increasing concentrations of the antimicrobial substance. This may be carried out by serial culture, or by culture on the gradient plate. The latter is prepared by allowing an agar medium containing the antibacterial substance to solidify at an angle in a petri dish and overlaying it with medium lacking the substance. The activity diffuses from the lower to the upper layer, giving a gradient in concentration on the surface of the medium from one side to the other. Bacterial cultures are streaked across it in the direction of the gradient, and, after incubation, the growth at the limiting concentration may be streaked into the higher concentration area, etc. Or, with some antibiotics, a series of agar mediums containing varying concentrations of the activity may be inoculated heavily, and the colonies growing out at various concentrations are resistant. Resistant variants may be produced *in vivo* by animal passage in the case of pathogenic microorganisms in experimental animals treated with subcurative amounts of chemotherapeutic agents.

Resistance may appear relatively rapidly, even in the first subculture, or more slowly over successive transplants. The maximum attainable may be of a low order, a few hundred fold or less in the maximum noninhibitory concentration of the activity, or many thousand fold. The degree of resistance and the rate of its emergence are, as indicated earlier, to a considerable extent characteristic of the microorganism and of the antimicrobial agent. The resistance so produced persists, to a greater or lesser extent, through successive subcultures in the absence of the chemotherapeutic substance.

For example, the development of penicillin resistance usually requires a considerable period of adaptation of the parent strain to increasing concentrations of the antibiotic. A resistance to several thousand fold the initial inhibitory concentration may be attained with staphylococci, but only of a much lower order, ten-fold to a hundred-fold with streptococci and pneumococci. In contrast, streptomycin resistance appears very rapidly, often on first subculture, in many kinds of bacteria and is of a very high order. In general, the higher the level of resistance produced, the longer it persists in culture in the absence of the antimicrobial substance. Thus penicillin-resistant streptococci revert relatively rapidly to the original sensitive state, but high resistance to streptomycin appears to be practically permanent.

Dependent strains. The occurrence of variants not only resistant to certain of the antimicrobial substances, but actually requiring them for growth, was observed first with sulfonamide and Neurospora and streptomycin and meningococcus and subsequently has been

found to occur with some frequency. Such variants are known as dependent strains.

The significance of this kind of variant lies in the fact that it suggests that some antibiotic substances function as specific metabolic inhibitors. There are two more obvious explanations of this phenomenon. One, the metabolic reactions of the microorganism have become so altered that the analogue has displaced the original metabolite in a metabolic sequence, and is now required. An alternative possibility is that the dependent microorganism produces antagonist (see below) in such large amounts that it is toxic, and the antimicrobial substance must be present to neutralize this toxicity and allow growth. The sulfonamide-*p*-aminobenzoic acid relation has been discussed elsewhere, and in this connection it is of particular interest that *p*-aminobenzoic acid acts as an antimetabolite on sulfonamide-dependent variants. A similar phenomenon has been described for the antimetabolite pyrithiamin. It is not known whether certain of the antibiotics, such as streptomycin, function as antimetabolites, but the occurrence of dependent variants suggests that this may be true.

When pathogenic microorganisms are made drug-dependent, they are unable to infect a susceptible animal host unless the animal is concurrently treated with the chemotherapeutic agent. Such drug-dependent strains have been of considerable interest as possible immunizing agents against infectious diseases, such as brucellosis, to which conventional heat-killed vaccines do not produce an effective immunity.

Cross-resistance. A strain of bacteria may be resistant to more than one kind of antimicrobial substance. When the history of the strain is known, as in the case of variants produced in the laboratory, it is clear that this resistance is of two kinds. One is multiple resistance in which a microorganism has been made resistant, one by one, to a number of antimicrobial substances. This is often encountered in bacterial strains, notably staphylococci isolated in resistant form. The other is cross-resistance in which a variant made resistant to one agent is found to have acquired resistance to another. This is almost uniformly true among the tetracyclines. Cross-resistance occurs only rarely between unrelated compounds and is of a low order. In relatively rare instances increased resistance to one agent may be associated with increased sensitivity to another.

Physiology of Resistance. Since the antimicrobial substances having therapeutic utility act by inhibition of growth processes, the basis of resistance to such substances is probably to be found in alteration of the metabolic processes of the microbial cell. The resistant variant may be presumed to differ in this respect from the parent strain, and the more obvious possibilities of the way in which this may occur are:

Synthesis of antagonists. The resistant variant may neutralize the activity of the chemotherapeutic agent by production of antagonists in greater than normal amount. This is clear-cut in the case of some sulfonamide-resistant variants. For example, many of the sulfonamide-resistant gonococcus and staphylococcus variants are resistant because they produce excessive amounts of inhibitor, often but not necessarily *p*-aminobenzoic acid. Similarly, diptheria bacilli made resistant to the inhibitory effect of the analogue pantolyl taurine synthesize pantothenic acid sufficient to support growth in the presence of the inhibitor.

It is doubtful how far this approach may be generalized. For example, all sulfonamide-resistant gonococci and staphylococci do not form increased amounts of sulfonamide antagonist, and some other mechanism is operative. In addition, as yet specific antagonists of many antimicrobial substances, especially the antibiotics, are not known.

Metabolism of the inhibitor. The antibacterial substance may be metabolized by the resistant variant. This is particularly well illustrated by the formation of penicillinase by penicillin-

resistant staphylococci. Penicillinase, which is formed as an adaptive enzyme by other bacteria, especially species of Bacillus, destroys penicillin activity by degrading it to the inactive penicillinoic acid. Resistance to pencillin can also be correlated with the formation of penicillin acylase, which acts by cleavage of the acyl group in penicillin G to give the relatively inactive 6-amino penicillanic acid.[40] The significance of metabolic destruction of penicillin by resistant strains of bacteria is underscored by the sensitivity of penicillin-resistant staphylococci to the synthetic sodium 6-(2,6-dimethoxybenzamido) penicillinate, whose structure differs sufficiently that it is not destroyed by penicillinase (Chap. 6).

This basis of resistance appears to be more or less restricted in that it has not been established as the basis of resistance to other antimicrobial agents. Even in the case of staphylococcus, while the naturally occurring penicillin-resistant variants are usually resistant because they form this enzyme, penicillin-resistant staphylococcus variants produced under laboratory conditions usually do not produce penicillinase.

Alternative metabolic pathways. A third way in which the variant may be resistant to an antimicrobial substance is by the development of an alternative equivalent metabolic pathway to replace that affected by the agent. The precise nature of the inhibiting effect is not known in most cases, but if such an alteration occurs, it might be expected to be accompanied by demonstrable physiological differences between the sensitive microorganism and its resistant variant. There is a great deal of evidence that resistance is accompanied by a variety of changes in metabolic processes such as altered nutritive requirements and impaired carbon metabolism.

For example, the assimilation of glutamic acid by staphylococcus described elsewhere (Chap. 3) in connection with the osmotic barrier, does not occur in penicillin-resistant variants, and free glutamic acid is not found in the cell, indicating that the variant has dispensed with the assimilatory mechanism and synthesizes its own glutamic acid. Consistent with this is the general observation that penicillin-resistant staphylococci which do not produce penicillinase no longer require as many preformed amino acids and bacterial vitamins. Among the enteric bacilli, the converse has been observed, *i.e.*, variants showing more complex nutritive requirements may be less sensitive to the antibacterial activity of penicillin than the parent strain.[104]

More specific changes have been found by a number of workers, especially Sevag and his co-workers.[57, 110, 116, 117] It has been shown, for instance, that pneumococci resistant to various chemotherapeutic substances have altered flavoprotein enzyme activity, possibly arising from a modification of the protein moiety, and reduced dehydrogenase activity and ability to oxidize glucose and intermediates such as lactate and glycerol. Similarly, streptomycin-resistant coliform bacteria partially or completely lose the ability to metabolize pyruvate anaerobically, and some variants partially replace the normal anaerobic phosphoroclastic metabolism of pyruvate, which is sensitive to streptomycin, with a streptomycin-resistant dismutative process. Pyruvate metabolism is altered also in chloramphenicol-resistant staphylococci so that it is not utilized but acts as a hydrogen acceptor to accumulate additional lactic acid in the medium.[101] Isoniazid resistance of tubercle bacilli is accompanied by a loss of the ability to produce catalase and a decreased virulence for the guinea pig, and the former may well be related to the observation that hemin antagonizes the antibacterial effect of isoniazid on tubercle bacilli.[32, 48, 80, 97] Similarly, indophenol oxidase–containing extracts of colon bacilli resistant to chlortetracycline have been found not to be inhibited by the antibiotic, while such extracts of sensitive bacilli are inhibited.[112]

While data such as these make it clear that the physiological processes of many bacteria are appreciably, or even profoundly, altered in resistant variants, available information is as yet too frag-

mentary to make it possible to discern a pattern in the physiological changes accompanying resistance.

It is not necessary to assume that a single mechanism is operative in the development of resistance to chemotherapeutic agents in that the foregoing are not necessarily mutually exclusive. In fact, the utilization of more than one mechanism in an apparently single adaptation is indicated in the observation that, in the adaptation of coliform bacilli to sulfanilamide there is first a reduction in the prolonged lag period interpreted as the development of an alternative metabolic sequence, followed by an enhanced growth rate attributable to the formation of an antagonist.

ADAPTIVE ENZYMES[31, 105, 106, 120, 123]

The ability of a microorganism to decompose a substrate may be very rapidly enhanced in the presence of that substrate. Although known for many years, this particular kind of bacterial variation has been the subject of renewed interest as *enzyme adaptation.* The enzymes which may be formed under the influence of the presence of substrate have been called adaptive enzymes in contrast with those which are formed whether or not the substrate is present, the constitutive enzymes. The formation of adaptive enzymes may be demonstrated by cultivation of the bacteria in a medium containing the substrate, and then testing the organisms for enzymatic activity, or a washed suspension of bacteria grown in the absence of the substrate may be mixed and incubated with it. In the first instance, activity is immediately apparent, the bacteria having formed the adaptive enzyme during growth, and in the second a latent period varying from 30 minutes to 2 to 4 hours occurs before rapid decomposition of the substrate begins.

A number of bacterial enzymes are adaptive in that they are formed wholly or in large part in the presence of the substrate. Among these, the β-galactosidase of the colon bacilli and penicillinase formation by *B. cereus* have been widely used as model systems. The amino acid decarboxylases are formed for the most part in the presence of the substrates. Many of the bacterial proteases are adaptive enzymes, for only small amounts of the enzymes are formed in the absence of protein, but filtrates from cultures in protein-containing mediums are actively proteolytic. Various other bacterial enzymes are adaptive also; thus, the enzyme responsible for the reduction of tetrathionate to thiosulfate by Salmonella, tetrathionase, and that catalyzing the reduction of nitrate to nitrite by the colon bacillus, nitratase, are adaptive as is the enzyme penicillinase formed by Bacillus species. The distinction between adaptive and constitutive enzymes may not always be a sharp one, for frequently, if not invariably, as in the case of the bacterial proteases, the adaptive enzyme is not completely lacking in the absence of the substrate.

The property of inducing adaptive enzyme formation is not necessarily confined to the substrate. Thus in the β-galactosidase adaptation of the colon bacilli, melibiose and galactose are inducers but not substrates, neolactose is a substrate but not an inducer, and thiophenyl-β-galactoside is a competitive inhibitor of the enzyme activity but is not an inducer. Lactose is, of course, both substrate and inducer in this instance. The adaptation, as assayed by the ability to ferment lactose, is more complex than this for it is now clear that the ability to ferment involves not only the β-galactosidase, but the cell must also be permeable to lactose. This last is a function of the enzymatic activity designated permease,[28, 30] which is also an adaptive enzyme (Chap. 3).

The question of whether or not the formation of an adaptive enzyme is associated with bacterial multiplication is one of considerable interest. In general it appears that some degree of multiplication is essential (cell division occurs even in suspensions of washed bacteria), and it has been shown in the formation of β-galactosidase by colon bacilli that the amount of enzyme was proportional to the number of new cells. In the ma-

jority of instances actual proliferation of the cells need not occur, though synthesis of the enzyme takes place during the latent period. The adaptive formation of formic hydrogenlyase by nonproliferating coliform bacilli requires a source of both energy and amino acids and is inhibited by assimilatory poisons such as nitrate and nitrite[82] and by the x-irradiation of the cells. In general, adaptive enzymes are formed under conditions of nitrogen starvation when there is no net increase in the number of cells, and this is probably a consequence of protein turnover in the nonproliferating cells.

It is both relevant and significant to note that substantially all, rather than a select few, of the cells subjected to the inducer-substrate participate in the formation of the enzyme. This, and the requirement for amino acids, indicates total synthesis of the enzyme. The adaptive utilization of maltose by yeast has, in fact, been found to be inhibited by amino acid analogues that also inhibit net incorporation from the amino acid pool.[60] The observed lag in the appearance of enzymatic activity is presumably due to activation of the enzyme, for total synthesis is very rapid; penicillinase, for example, is formed within 30 seconds. Adaptive enzyme formation is inhibited by growth-inhibiting antimicrobial substances and this is taken to suggest that these substances inhibit synthesis of constituent protein by the bacterial cell. It is of interest in this connection that kinetic considerations have shown that at least in the adaptive formation of penicillinase by *Bacillus cereus*, the inducer-substrate, penicillin, cannot be incorporated into the enzyme but must function catalytically in that each mol of penicillin fixed or destroyed gives at least 10 mols of the enzyme (Pollock[105]).

The phenomenon of enzyme adaptation has been made use of, especially by Stanier, in the elucidation of adaptively controlled metabolic pathways. In a chain reaction controlled by adaptive enzymes represented by:

$$\underset{E_a}{A \rightarrow} B \underset{E_b}{\rightarrow} C \underset{E_c}{\rightarrow} D \underset{E_d}{\rightarrow} \text{etc.}$$

the reaction A→B is catalyzed by the adaptive enzyme E_a, B→C by enzyme E_b, etc. When any intermediate is supplied, the chain reaction continues to completion since the product of one reaction is the substrate for the next, and in its presence the appropriate adaptive enzyme is formed. Thus substances presumed to be intermediates can be tested for actual participation in the chain reaction; *i.e.*, if the compound is dissimilated immediately by cells adapted to substrate *A*, for example, but not by cells not adapted to *A*, it is highly probable that it is an intermediate. This technique has been applied, in whole or in part, to analysis of adaptive patterns in the oxidation of Krebs cycle components, aromatic compounds including tryptophan, tyrosine, purines, etc.

RECOMBINATION[83]

The possible occurrence of sexuality among bacteria and related microorganisms has permeated the interpretations of variation phenomena for many years, but it was not until 1947 that unequivocal evidence for the recombination of marker characteristics, and inferentially conjugation, between unlike cells was presented by Lederberg. In essence this consisted of the isolation of radiation-induced variants of the parent K-12 strain of coliform bacilli, characterized by physiological differences such as requirements for specific amino acids. When such variants are grown in mixed culture, progeny may be isolated in which the markers occur in the possible combinations, including combinations not present in the original strains, and in kind and proportion to be expected on the assumption of a conjugative recombination.

The distribution of marker characteristics in the recombinants is not invariably random in that apparent crossing and linkage occur to disturb the random distribution to be expected on the assumption that the markers are independent. It has therefore been possible

to map, to a certain extent, the loci of determinants with respect to one another.[27, 37] A number of terms have been coined for definition of the various spatial relationships. A *recon* is a unit of nucleic acid not subdivisible in recombination, and consists of about 12 nucleotide pairs. A *muton* is the shortest length of the nucleotide chain which must be altered to produce a mutant, a maximum of about five nucleotide pairs. Finally, a *cistron* is the unit of length, about 4000 nucleotide pairs, required to produce some kind of integrated function, but function can be impaired by mutations at many different locations on a cistron.

Recombination has also been demonstrable between bacteria, not only of different species, but between genera. This was first described as occurring between coliform bacilli and dysentery bacilli (Escherichia and Shigella), and subsequently recombination between coliform strains and a variety of Salmonella species, including the typhoid bacillus, has been shown to occur, both *in vitro* and *in vivo*.[12, 13, 95, 113, 143] The occurrence of different bacterial genera, Escherichia and Shigella, within the same fertility system casts serious doubt on the criteria used for the differentiation and characterization of bacteria, not only on the species but also on the generic level (Chap. 8).

Bacterial Sexuality.[74] It has also become clear that "male" and "female" types of bacterial cells occur that are demonstrably different. The "female" cells have a higher stability in suspension and are optimally agglutinated at a lower pH than are "male" cells, thus suggesting surface differences, and "female" cells also have a somewhat greater affinity for basic dyes.[144]

The "male" cells appear to be of two kinds, designated F+ and Hfr. The F+ cells donate the F+ capacity to F—, or "female," cells with high frequency, but recombine with F— at low frequency. Conversely, while Hfr cells cannot convert F— cells to F+, they combine with F— cells with a high but variable frequency, depending upon the markers used. In the recombination between Hfr and F— cells, Hfr occurs only rarely in the recombinants, and thus tends to be eliminated. Conjugation probably involves appropriate receptor sites, or their equivalent, and the inhibition of mating by periodate, by temporary destruction of the virility of F+ cells, suggests that some surface polysaccharide-containing substance is involved.[119]

The transfer of genetic material between cells takes place across a bridge, and the complete transfer takes about an hour. If the conjugated cells are agitated, as by treatment in a blender, before transfer is complete, partial transfer is found in the progeny. This allows the determination of a sequence of hereditary units that supplements that deduced from evidence of linkage.[64]

Viral Recombination. It is of particular interest that recombination occurs between variant or closely related strains of viruses in that these agents appear to be genetic mechanisms uncomplicated by an associated physiological system. The bacterial viruses of the T series of coliphages and influenza viruses have been the most extensively studied, but there is evidence that this phenomenon occurs among other viruses such as those of herpes simplex and poliomyelitis.

Recombination between viruses occurs when sufficiently closely related strains or variants are "grown" together as mixed infections of the same host cell. As pointed out elsewhere (Chap. 4), mixed infection rather than interference is produced under appropriate experimental conditions. The progeny resulting from such mixed infections are quite analogous to those produced by bacterial recombination, *i.e.*, types appear that are combinations as well as replications of the strains making up the inoculum. The observation that lysogenicity, *i.e.*, apparently latent infection of the bacterial cell with temperate strains of bacterial virus, also behaves as a stable genetic character in recombination experiments with K-12 and B coli-

form bacilli and the λ coliphage serves to link the phenomenon of recombination with that of transduction (see below).

There seems to be no morphological aspect of viral recombination in that the mature virus particle does not exist during active multiplication within the host cell, and conjugation of vegetative virus resolves to an interaction between two kinds of viral nucleic acid. The nature of the exchange of hereditary elements is best understood for certain of the bacterial viruses, using markers such as minute plaque formation, ultraviolet sensitivity, host specificity, and rapidity of lysis. Among these viruses the "mating" that gives rise to recombinants is, in effect, multiple in that the primary redistribution of genetic material is followed by other matings between recombinants and thus resembles recombination between populations rather than individuals. The occurrence of such successive matings is indicated by the appearance of progeny which could not have been formed by any kind of exchange between the two parental viruses.[18, 19, 125]

Analogous recombination occurs among several of the animal viruses, and of these the best known is influenza virus, largely through the studies of Burnet and his co-workers.[11,20,51,52,72,90,91,103] The markers are characters such as pathogenicity for the mouse lung, for the embryonated hen's egg, neurotropism, and antigenicity. The results of recombination experiments have shown, not only that recombinants occur, but that there are linkage groups, as for example between mouse lung pathogenicity and antigenic character, and, further, that noninfectious virus inactivated by ultraviolet irradiation may enter into combination with infective virus. This last is suggestive of the transformation phenomenon (see below), possibly implying that the latter may be a special case of the recombination phenomenon. Recombination with the production of hybrids also occurs among the pox viruses[42, 43, 137] and between antigenic types of poliovirus.[122]

TRANSDUCTION[29, 63, 84, 85, 142]

The method of transfer of genetic material from one cell to another based on a sexual system and involving recombination of entire blocks of genes in intact cells is the conventional one in that it is shared by all kinds of living organisms, including microorganisms. In addition to recombination, there occurs among the microorganisms a transfer of genetic fragments, mediated by bacterial viruses, or introduced into the cell directly as macromolecules of polymerized nucleic acid. In either case the process is one of transduction of genetic elements, but the term transduction has been applied to the phage-mediated transfer, while the direct application of nucleic acid has been called transformation because it was first observed in the transformation of immunological types.

In phage-mediated transduction the genetic material is transferred from the donor cell to the recipient by bacteriophage. Virulent phage may act as a transducing agent under appropriate conditions, but most often the transduction is by temperate phage. The lysogenic donor cells containing the marker are broken down by induced phage lysis, recipient cells are infected, and the marker is present in those which survive by becoming lysogenic. This is studied in the most part as transduction between strains of a given bacterial species, or different species, but it also occurs, like conjugation, across generic lines, between coliform bacilli and dysentery bacilli.[94]

Two kinds of transduction are to be distinguished. The one, sometimes called generalized transduction, is causal in the sense that the fragment of donor cell genetic apparatus carrying the marker is injected into the recipient together with viral DNA, but is not a part of it. About three per million phage particles are transducing for the marker, or about one transducing particle for every 300 to 500 bacterial donor cells lysed, so relatively few of the lysogenic recipients are found to contain the marker. It is characteristic of transduction that usually only a single

marker is transduced because the transducing particle contains only a genetic fragment from the donor cell, but occasionally markers may be so closely linked that both occur in the same fragment.

The markers may be of various kinds. Variants may be isolated which are characterized by markers such as specific nutritive requirements, fermentative abilities, resistance to chemotherapeutic substances, or antigenicity. Lysis of the lysogenic variant is induced, as by treatment with ultraviolet light, and the marker characterizing the variant is transferred together with lysogenicity, to a susceptible strain of bacteria. In this way a drug-sensitive strain of bacteria may be made drug-resistant, or, by transduction of antigenicity, new serotypes may be created.

The essentially passive role of the bacterial virus is substantiated by the ability of ultraviolet-irradiated phage to transduce, *e.g.*, infectivity is destroyed by radiation more rapidly than the transducing factor, and transduction may be accomplished without inducing lysogenicity in the recipient. The virus thus functions as only a carrier of genetic fragments resulting from the lysis of the donor cells, and as a means by which such fragments are introduced into the recipient cell. The fragment suffices to replace the homologous gene in the recipient, the exchange occurring quite soon after the fragment enters the cell, and the prior homologue does not reappear. Such homologous replacement, as for example of flagellar antigens of Salmonella, suggests an organized genetic system in the recipient bacterium but offers no clue as to how the homologues are associated prior to the elimination of one or the other.

The other kind of phage-mediated transduction derives from the intimate relation between prophage and the host bacterium in the lysogenic state. Although autonomous as in infectious particle, the temperate prophage becomes a part of the bacterial genetic apparatus, and the induction of lysogeny is, in effect, a transduction. In recombination experiments the prophage segregates as if it were a bacterial marker and, further, it may be transduced as prophage by another bacterial virus. The λ coliphage is linked with galactose fermentation, suggesting that in this case the phage may occupy a predetermined site in the bacterial genetic apparatus. The fact that lysogenicity may be multiple, to as many as three different prophages, shows, however, that more than one site may be occupied by prophage. It has been suggested that the genic factors so imposed on the genetic apparatus of the host cell are not always a part of that apparatus and may be regarded as episomes.[73]

Changes correlated with lysogenic conversion differ from those of generalized transduction in that they are present in all, rather than a small portion, of the lysogenic progeny and cannot be dissociated from the presence of prophage. The most interesting of these is the toxigenicity of lysogenic diphtheria bacilli.

It appears to be definitely established that all toxigenic diphtheria bacilli are lysogenic, though all lysogenic strains are not necessarily toxigenic, and that the induction of toxicity is inseparable from phage. This phenomenon has been studied in detail by Groman[10, 58, 59] and may be described briefly. When any strain of diphtheroid bacilli sensitive to phage β is made lysogenic, it invariably becomes toxigenic; when lysogenicity is lost toxigenicity disappears but may be induced again when the strain is again made lysogenic. Another strain of phage, γ, differing in host range, produces lysogeny of sensitive strains of bacteria, but these are not toxigenic. Recombinants of β and γ prophages have been isolated, of which one, β', has the host range of β but does not produce toxigenicity, while another, γ', has the host range of γ and does produce toxigenicity. Further, bacterial strains doubly lysogenic for β and γ are toxigenic. Thus the prophages behave as if they were bacterial genes, with toxigenicity dominant, affecting the metabolic processes of the bacterium concerned with the production of the

iron-deficient porphyrin complex that is diphtheria toxin.

Transformation[7, 41, 71]

Transformation is a transduction accomplished by direct treatment of the recipient cell with nucleic acid from the donor cell and thus relates to viral replication via infectious nucleic acid (Chap. 4). It was first observed with pneumococcus in experiments in which the immunological type, determined by the antigenicity of the polysaccharide capsular substance, was changed or transformed. It also occurs with respect to characters other than antigenicity on the one hand, but, in addition, is to be distinguished from other kinds of immunological changes, such as those associated with the S–R dissociation, environmental modification of antigenicity such as the temporary repression of flagella and flagellar antigen formation in the presence of dilute phenol.

It was suggested by Theobald Smith and Reagh in 1904 that bacteria might be immunologically altered by residence in different hosts, although they were unable to demonstrate such changes, and much later Holtman[68, 69] showed that enteric bacilli could acquire the antigenic specificity of Forssman antigen by growth in the presence of this substance, the specificity persisting through twenty to fifty transplants of the modified bacilli. The latter probably accounts for the irregular occurrence of Forssman antigen in a variety of bacteria and the presence of Forssman antibody in presumably normal animals, such as rabbits, in which the antigen does not occur, as a consequence of infection with such bacteria.

The variation now known as transformation was described by Griffith in 1928 who inoculated animals with avirulent R strains of pneumococci originally derived from, for example, type 1 pneumococcus, but no longer having type specificity, together with a killed vaccine of encapsulated, type-specific pneumococcus of another type, such as type 2. Following development of an infection, living virulent pneumococcus of the vaccine type, in this example type 2, were found, indicating that the R variant from type 1 had been converted, or transformed, to the S virulent encapsulated type 2, *i.e.*, had acquired the ability to synthesize the type-specific capsular polysaccharide of the vaccine strain. This phenomenon was subsequently studied in detail by others, especially Avery and his coworkers, and could be made to occur *in vitro*.

The Transformation Reaction. Transformation *in vitro* occurs in several distinct stages. There is first a preliminary period of growth in an appropriate medium (see below); second, a period during which transformable cells make their appearance, usually during the latter part of the logarithmic growth phase; third, a short period, perhaps 10 minutes, during which the transforming principle is taken up by the recipient cells; fourth, a longer period, 45 to 60 minutes, during which phenotypic expression of the transformation develops; and, last, the appearance of progeny of the transformed cells which show the acquired character and breed true indefinitely.

Transformation Factors. There are three variables in this process. The first is the substance originating in the donor cells and transmitted to the recipient, the transforming principle or TP. This was first isolated from pneumococcus, and subsequently from other bacteria, as a highly polymerized deoxyribonucleic acid; depolymerization or small changes such as a minor amount of deamination result in inactivation. The material retains its activity during purification and fractionation, as in resin columns, and appears to have a molecular weight of about 700,000, though there is reason to believe that only a portion of the macromolecule is active. Only a fraction of the TP is irreversibly adsorbed by the treated recipient cells, and most of it can be washed off or decomposed by deoxyribonuclease without interfering with the

transformation. Kinetic studies indicate that one molecule per recipient cell is sufficient to transform, but when TP is present in concentrations greater than ten molecules per cell, the rate of transformation is less than the extrapolated rate, suggesting some kind of interference.

The second variable is known as the serum factor which is, of course, present in *in vivo* transformation, but which must be supplied in the medium when transformation occurs *in vitro*. This factor is differentiable by dialysis into two fractions. The dialyzable fraction is replaced by pyrophosphate, and the nondialyzable fraction by bovine serum albumin. The manner in which these factors function is unknown. In some kinds of transformation a third component of the serum factor, agglutinating antibody to the bacteria, is required, but this is a special case in that it is required only in certain immunological transformations.

The third variable is the susceptibility of the recipient cells to the action of the transforming principle. As implied above, all the cells in a bacterial culture are not capable of accepting the transferring principle. Cells which are, are said to be competent, and competent cells appear later in the logarithmic growth of the usual culture. The proportion is not high, ranging from one to ten per thousand cells. They occur in peaks, and under some conditions these peaks occur periodically. The peak persists in time only 15 to 20 minutes and is apparently associated with maturation of the individual cell; the peaks and their periodicity are a result of a partial synchronization of cell division. Cell division may be approximately synchronized[23] (Chap. 4), with increase in the proportion of competent cells to as much as 17 per cent. That competence is associated with a physiological state is indicated by the occurrence of transformation when TP is not present in the growth medium but on transfer of competent cells to a medium containing TP. Further, competent cells may be frozen and still retain competence for as long as 6 weeks, though viability may persist for 18 months when the cells are stored at —20° C.[50]

Other Transformations. Transformation is not limited to that of pneumococcal types but has been demonstrated for immunological character in Hemophilus, meningococcus, coliform bacilli, and Shigella. Characters other than antigenicity are transformable also, including such diverse properties as fermentative abilities and drug-resistance. In fact, it may be supposed that almost any bacterial characteristic may be reproduced by transformation. Transformation may also occur across species, or even genera if the pneumococcus is to be regarded as so separated from the streptococci, for streptomycin resistance has been exchanged between these microorganisms, in both directions via soluble DNA.[16]

Fibroma-myxoma.[76] A closely similar, if not basically identical, phenomenon was described in 1936 by Berry and Dedrick, and is known as the Berry-Dedrick phenomenon or as the fibroma-myxoma virus transformation. The fibroma and myxoma viruses are tumor viruses infecting the rabbit, and belong to the pox group (Chap. 37). The former produces a benign, localized tumor which begins to regress within a few days, while the latter produces a fulminating rapidly fatal infection. The transformation was originally described in substantially the way Dawson described type transformation of pneumococcus *in vivo*. Myxoma virus may also be transformed into fibroma virus in tissue culture. The transforming principle is effective not only as a heat-inactivated, DNAse-resistant preparation of fibroma virus, which electron micrographs indicate to be vegetative virus, but also as a urea-solubilized preparation which is sensitive to DNAse.

There is evidence that this phenomenon may be a general one, at least among the pox viruses, for Japanese workers have reported similar transformations in tissue culture of myxoma virus into vaccinia virus and ectromelia (mouse pox) virus, and of ectromelia virus into vaccinia virus.[61, 62]

THE NATURE OF MICROBIAL VARIATIONS

It is clear from any consideration of microbial variation that all, or nearly all, of the characteristics made use of in the differentiation and identification of these forms are subject to alteration. While many of them do not yet have counterparts in classic genetics built around differentiated higher organisms, there is no reason to believe that other than substantially the same mechanisms are operative for all living things.

At the same time, studies of microbial variation have broadened tremendously concepts of genetic mechanisms, and it has become clear that variability need not be the random process of selection of the fittest among spontaneously arising genic mutants, but may, in fact, be induced in the broad sense. The facility with which inductive or seemingly inductive changes may occur is possibly related to the more intimate association of the germ cell of the microorganism with environmental influences as contrasted with the protection of the barrier of somatic cells intervening between the environment and germ cells of higher forms.

Further, the concept of virus as an independent gene or group of genes, tentatively suggested much earlier, tends to be substantiated with the demonstration of situations, such as lysogeny, in which it becomes difficult to formulate definitions of virus and gene which are mutually exclusive. It is equally significant that parasitism is here extended to a genic level with extinction of the autonomy of the two agents.

It may be taken as fully established that the differential characters of microorganisms are the phenotypic expression of the functioning of a genetic apparatus whose component parts behave as discrete entities, which may or may not be linked, and thus have the attributes of genes of classic genetics. It is equally well established that many differential characters depend, at least for their expression, on the presence of a specific substrate or other inducer in the environment. The question at hand is that of determining which of these mechanisms, or modifications of them, are altered with the appearance of microbial variants, and their relative importance in the phenomena of microbial variation. In general, there is a tendency to subordinate one or the other, and to take the view either that genic mutation is relatively all-important or that the great majority of microbial variants represent an adaptation in the sense of a response to environmental induction.

MUTATION

It is already apparent from foregoing considerations that genic changes may be induced in microorganisms in the phenomenon of transduction, either phage-mediated or direct as in transformation. The definition of mutation must inevitably be broadened from its classic conventional meaning to include these kinds of induced genic modification. So it is not a matter of whether genic changes occur, but rather one of, first, whether spontaneous undirected mutations occur and, second, the extent to which microbial variants represent a result of this process.

Spontaneous Mutation. Randomness is the identifying characteristic of spontaneous mutation in that the mutation occurs as a result of the operation of an indefinitely large number of small independent causes rather than one, or at the most a few large and important causes. The necessity for studying microorganisms as populations requires that a spontaneous mutant be selected so that it may become predominant and characterize the population in order to be detectable. It is an unfortunate consequence of this that a selective environment, whose selectivity is dependent upon the presence of a substrate or other potential inducer, is interposed at one point or another. The oc-

currence of spontaneous mutation must be inferred from population behavior* and cannot be of an unequivocal nature, but when such evidence is derived in different ways, its cumulative weight becomes great.

Many bacterial variations behave as if they were a consequence of spontaneous mutation, and the mutants selected, rather than induced, by the culture medium. For instance, the frequency with which variants appear is of the same order as the mutation rates established in higher forms, *e.g.*, one variant cell per 10^6 to 10^9 parent cells. This rate may be increased by treatment with radiation or other mutagenic agents such as nitrogen mustards (β-chloroethylamines), though not with colchicine. These variants breed true, in fact could not be found if they did not, and behave in recombination experiments as genic entities.

If such variants occur spontaneously, it follows that their number is directly related to the size of the population, and in small populations would fluctuate widely. This consideration is the basis of the fluctuation test devised by Luria[93] in which a parent population sufficiently large to contain some mutant cells is divided into many small parts which are grown independently. If only an occasional mutant is present in the parent population not exposed to the selective environment, its appearance in subcultures in the selective medium should fluctuate erratically rather than appear uniformly. Many microbial variants do show such fluctuation. The criticism that the fluctuation test shows only randomness, not necessarily spontaneous mutation, is, of course, a legitimate one.

A more direct method of demonstrating the occurrence of mutation in the absence of possible inducing substrates, *i.e.*, pre-existence of the mutant relative to its contact with the selective environment, is the replicate plating method devised by Lederberg.[87] A plate culture on a nonselective medium is allowed to grow for a short time to produce microcolonies. The growth is then impressed on the surface of sterile velveteen, and the pattern of growth is imprinted on plates of selective mediums. Following incubation the growth patterns and the colonies on a selective medium, as for example one containing an antimicrobial substance, are compared, and if the colonial growth on the selective medium corresponds to that on the nonselective medium, it is inferred that the variant clone was pre-existing with respect to the selective agent.

Furthermore, the occurrence of conjugation among the microorganisms with consequent recombination of markers shows that such markers, gene-controlled enzyme systems,[139] segregate in a manner completely consistent with the occurrence of their determinants in a linear arrangement. Too, as indicated earlier, the process of transfer of genic material during conjugation may be interrupted to show a sequence in the markers transmitted. In consequence, it has been possible to prepare maps of gene loci for bacterial viruses as well as bacteria,[37] but the necessary detailed information is not as yet available for the animal viruses.

The cumulative weight of this kind of evidence is considerable, and is regarded by many as establishing beyond reasonable doubt the selection of spontaneous mutants as the basis of the great bulk of microbial variations.

Its application to the phenomenon of drug resistance may be taken as a representative example.[36] Suppose Bacillus X is adapted to grow in the presence of an antimicrobial agent in concentrations which inhibit the growth of the parent strain. The nature of the resistance, *e.g.*, the development of alternate metabolic pathways, decomposition of the drug, etc., is, of course, irrelevant here. The process of adaptation is conceived as the spontaneous appearance in the parent population of resistant mutants which grow in the concentration of the drug applied while the parent strain does not. Resistance may be increased, as described earlier, by cultivation of the variant in successively higher concentrations, and in each of these new mu-

* For a detailed discussion of the relevant statistical theory see references 5 and 6.

tants appear spontaneously which are characterized by an even greater drug resistance until some maximum is reached. This is the stepwise rather than uniform rate of emergence of resistance which is regarded as additional evidence of the spontaneous, rather than induced, appearance of resistant mutants.

On continued culture in the absence of the drug, resistance disappears gradually at about the same rate that it was acquired, and this is interpreted as a consequence of a series of back mutations to sensitivity to the drug.

INDUCED ADAPTATION

The wide range over which microbial adaptation occurs, and the specificity of the adaptations, has suggested to many workers that the adaptive process is a directed one rather than a chance occurrence, that is to say, is induced by the chemical character of the environment, rather than a consequence of the selection by the environment of spontaneously occurring variants. Further, the usual dependence upon the presence of the substance toward which the adaptation is directed suggests that the relation between the two is causal rather than coincidental. There is no question that specific induction occurs in the phenomenon of enzyme adaptation described earlier, and it is established that the synthesis of enzyme is specifically induced, at least in the sense of being markedly stimulated, in the presence of the substrate. The theoretical aspects of this phenomenon, upon which any generalization is dependent, have been examined by Yudkin[140] who has formulated the concentration relationships of enzyme and substrate in mass action form. This approach has been further elaborated by Mandelstam,[96] and shown to fit closely with the observed behavior of these systems.

The physiological processes of the metabolizing cell may be regarded as a series of interrelated reactions in which the product of one is the substrate of another, as in the catalysis of respiration. Or the linkage may be cyclical, one of the later reactions in the sequence providing an intermediate functioning in one of the earlier reactions in conjunction with some other substrate, as in the cyclical processes of carbohydrate metabolism such as the alcoholic fermentation and the Krebs cycle. The individual reactions are determined by the concentration of intermediates, the amount of enzyme, and the reaction velocities.

Enzyme Balance. If the kinds and proportions of enzymes present within the cell are affected by the nature and concentration of substrates present, adaptation can consist of an adjustment of the cellular physiology as a whole to utilization of the substrates with maximum efficiency. This is, in effect, a generalization of the phenomenon of enzyme adaptation and has been most fully developed by Hinshelwood.[66] From this point of view, the sequence of metabolic events can be visualized as follows. A steady state occurs during logarithmic growth, but with the exhaustion of food materials, accumulation of metabolic products and the like, there is cessation of the maximum growth rate, concentrations of diffusible intermediates fall, and the individual enzymes decay and alter in their relative proportions as the system moves toward a new equilibrium. On transfer to fresh medium, readjustment is initiated during the lag period with the building up of concentrations of intermediates and a synthesis of enzymes in a shift toward an equilibrium consistent with maximum growth rate. Thus, the relative proportions of the elements of the catalytic system and the concentrations of substrates of the individual reactions exist in a series of metastable states of enzyme balance under ordinary culture conditions.

There is a great deal of evidence that alterations of this kind occur. For example, the formation of bacterial deaminases is suppressed during rapid growth in the presence of fermentable carbohydrate and does not occur at acid reactions but decarboxylases are formed a:

an acid reaction, and both kinds of enzymes are formed in the later stages of culture growth; the kind and proportion of enzymes present are a function of the age and condition of the culture. Similarly, the products of carbohydrate metabolism depend upon environmental factors, again a matter of balance in the function of the cellular enzymes.

If a strain of bacteria is transplanted to a medium differing from the one in which it has been grown, a greater degree of adjustment occurs. For instance, coliform bacilli ferment both glucose and lactose, but if cultured continuously on glucose and then transferred to lactose, the growth rate, measured as the mean generation time, is reduced. After several transfers in lactose the rate increases, and the strain grows as rapidly in lactose as it formerly did in glucose. If the training to lactose is only partial, reversion occurs in nonlactose-containing mediums, but if the strain has been carried through many transplants in lactose, the ability to use the sugar with a maximum efficiency persists. Since the bacterium ferments both sugars with facility the adaptation is not overt, but it is, nevertheless, a typical adaptation, and the result is clearly a consequence of alteration in the balance of enzymes already existing in the cell. The adaptive process is much more striking, and less obviously a result of change in enzyme balance, when there is a great differential between the parent and adapted strains, as between no detectable acidity and an active fermentation, whether it occurs slowly on successive transfer or very rapidly as in the formation of an adaptive enzyme.

The change in enzyme balance may be quantitative in nature. The enzyme in question may be present only as a precursor, the formation of enzyme occurring in the presence of substrate through mass action as suggested for adaptive enzyme formation. Or the enzyme may be initially present in only very small amount, but it has a high turnover rate. Or, finally, catalysis of the new reaction may be a function of an existing enzyme but at a relatively low reaction velocity, and adaptation a matter of expansion of that enzyme. The last opens the question of qualitative modification of the enzyme, in that distortion of its specificity by a slightly different substrate requires greater activation energy and hence a lowered reaction rate, but if the substrate is present during formation of the enzyme, and the distortion not too great, a modified pattern might eventually result.

Returning to the example of the development of drug resistance by microorganisms, it is as satisfactorily accounted for on the basis of an adjustment of bacterial enzyme systems as on that of a stepwise selection of resistant mutants, and here the nature of the resistance becomes significant. As indicated earlier, drug resistance may result from the production of an inhibitor such as *p*-aminobenzoic acid in excess amounts, a qualitative modification such that the drug displaces the antagonist as an essential metabolite, or by diversion to an alternate metabolic pathway. The first of these is clearly an expansion of an existing catalytic system. Regarding the second, suppose it be assumed that the drug displaces the prosthetic group of an enzyme, *viz.* pantolyltaurine, to give a modified enzyme and the formation of different metabolic products. The adaptation can thus consist of an altered enzyme balance to allow the metabolism of the new intermediates, and, when this is established, the original essential metabolite competitively inhibits the new system, and the drug has become an essential metabolite. The development of an alternate metabolic pathway is likewise an expansion of an existing enzyme or system of enzymes which ordinarily contributes in but small amount to the maintenance of the concentration of a given intermediate, perhaps because of lower reaction velocity. When the function of the usual system is inhibited by the drug, the alternate is expanded, perhaps only quantitatively, to neutralize the effects of the lower reaction velocity. The whole is analogous to an industrial system in which a raw material for a given process is shut off, an alternative, more ex-

pensive, process is developed, and the economies effected in large-scale operation make it as efficient as the original process.

There is considerable evidence in support of the view that the development of drug resistance is intimately related to the presence of the antibacterial substance which functions to distort metabolic processes[115, 118] as well as to serve as a selective element in the environment. For example, resistance may develop in the presence of concentrations of the drug too small to inhibit growth, or the rate of development of resistance may depend upon the way in which the microorganism is exposed to it or be too rapid to be accounted for by selection of spontaneous mutants. Such evidence, too detailed for presentation here, is not only consistent with a relatively rapid adjustment of the bacterial enzyme systems to the new circumstances, but in some cases hardly admits a different interpretation.[34, 138]

Induction versus Mutation. There is probably little or no justification for regarding specifically induced microbial variation as completely apart from, and competitive with, variation arising as a consequence of alteration in the genetic apparatus of the microorganism. Conversely, it is untenable to account for all microbial variations other than adaptive enzyme formation on a basis of spontaneous genic mutation and selection. Rather, it is self-evident that the observed variant is the phenotypic, and therefore environmentally dependent, expression of the microbial genotype.

In relatively few instances, notably that of the β-galactosidase-permease system of mutabile strains of coliform bacilli described earlier, can these be sharply distinguished. In the great majority of microbial variations the distinction is not clear, leading to uncertainty as to the nature, or more precisely the relative importance, of the mechanisms operative in the production of the observed variant. The induced variations combine with genic mutation to increase the adaptability of the microorganism substantially beyond the limited range imposed by a one-to-one or similar relation between genic unit or subunit subject to spontaneous mutation and the production of, say, a given enzyme. By their occurrence, the induced variations make unnecessary the postulation of improbably complex polygenic controls.

The lack of clarity of distinction goes beyond that of interpretation of a given variation for, on the one hand, genic modification can be induced in the transduction reactions, and, on the other, metabolic sequences may behave in a unitary way suggestive of a genic nature. In connection with this last, Pollock[105] has suggested that chain reactions controlled by adaptive enzymes may be cyclic in that the end product and the starting material are identical. Such self-reproducing biochemical cycles, or "genocycles," may well have some degree of autonomy at a subcellular level, with single unit cycles showing the characteristics of genes, and multiple unit cycles that of linked genic characters. Further, such a view includes the possibility of variation arising from intracellular competition between cycles through diversion of a component of one cycle to another or even the initiation of new cycles by rearrangement to give the appearance of genic mutation.

MICROBIAL PHYLOGENY

Among the microorganisms, as among other living things, phylogenetic or evolutionary relationships are inferred from existing, *i.e.*, persisting, forms. At one time it was thought that bacteria were the first forms of life because only the autotrophic bacteria could live in an inorganic environment. It is hardly likely that a complete, highly organized cell, containing complex catalytic systems and

having the broadest synthetic powers, could have appeared *de novo* in an inorganic world.

There is now excellent reason to believe that a chemical evolution, resulting in the appearance of organic matter, antedated the origin of life.[15, 49, 53, 100, 129] It was suggested by Haldane in 1926 that amino acids and heterocyclic compounds found in protoplasm could have been formed from single carbon compounds such as formaldehyde under the influence of solar ultraviolet light. The indicated experiments have been carried out by many workers and have shown that such organic compounds may be formed in this way. Some years later Oparin suggested that carbon was initially in the form of metallic carbide which, on contact with water, formed acetylene, and polymerization of the acetylene, catalyzed by mineral substances, could have resulted in the formation of long-chain carbon compounds which may, in turn, serve as precursors of the kinds of molecules found in living protoplasm. A third possibility is that of the formation of organic molecules from inorganic substrates under the influence of high energy radiation, and this has also been substantiated by experiment. Finally, electrical discharges in the presence of substances such as methane, hydrogen, ammonia, and water, *i.e.*, under reducing conditions, result in the appearance of a variety of compounds, particularly of the amino acid type; these reactions too are demonstrable in the laboratory. The appearance of such substances through any or all of these means is necessarily a random process, but as soon as a substance occurs that has catalytic activity, an element of selectivity appears, and a plausible case may be made for, for example, the chemical evolution of the iron porphyrin compounds.[4, 21, 22, 98]

Presumably toward the end of this period of chemical evolution, self-reproducing substances appeared to constitute the beginning of the phenomena uniquely associated with life. It has been suggested by Haekel, Oparin, and more recently Salser,[111] that the occurrence of autonomous biochemical cycles, perhaps analogous to the phenomenon of enzyme adaptation, preceded the development of the coding mechanism of replicating nucleic acids. It has been objected that a nucleic acid system is, in fact, not only a necessary, but a much more probable primitive system,[99] especially in view of recent information on the formation of nucleotide chains in the absence of the living cell. However this may be, it is likely that the bacteria and blue-green algae were among the first forms of life organized on a cellular level. Of existing physiological types of bacteria, the facultative anaerobic free-living heterotroph would appear to most nearly resemble an ancestral form from which four main lines of divergence to more highly specialized forms may be distinguished.

A relative scarcity of metabolizable organic substrate would favor the evolution of the autotrophs, and these may have differentiated quite early, first as the facultative autotrophs and then the obligate autotrophs. The facultative autotrophs are characterized by a relative flexibility in their requirements for oxidizable substrate and retain to some degree the ability to respire anaerobically. The sulfur bacteria, oxidizing compounds of sulfur ranging from hydrogen sulfide to polythionates and able to live anaerobically in the presence of nitrate, are representative of the group. Such bacteria may be regarded as transitional forms in the phylogenetic sense, giving rise on the one hand to the pigmented photosynthetic sulfur bacteria, and on the other to the obligate aerobic obligate autotrophic bacteria such as the nitrifying bacteria restricted to the oxidation of ammonia to nitrite or nitrite to nitrate.

Two lines of divergence occur with respect to the utilization of gaseous oxygen. One of them is that of the obligate anaerobes, highly specialized free-living heterotrophs closely adapted to living in the absence of oxygen, which lack a portion of the respiratory catalytic system, and characterized by unusual energy-yielding reactions such as the oxidation-reduction of paired amino acids and the

metabolism of lactic acid to compounds such as butyl alcohol and acetone. The other line is that of the obligate aerobes which grow poorly or not at all in the absence of molecular oxygen since they lack the ability to reduce many substances other than oxygen in the oxidation-reduction reactions of respiration.

The parasitic and pathogenic microorganisms may have been derived from these three groups of free-living heterotrophic bacteria. It is probable that toxigenicity in the broad sense is a prerequisite to the evolutionary development of the parasite in that it represents the entering wedge to invasion through deleterious effects on the integrity of the host tissues. The formation of potent toxins by certain of the free-living heterotrophs such as the botulinus bacilli, and the limited invasive powers of highly toxigenic bacteria such as the tetanus bacillus, suggest that they are forms which, though toxigenic, are not yet, or are only poorly, adapted parasites in that they are unable to invade the host tissues and set up a sufficiently mild infection so that they may persist in the host.

There appears to be little question that the host-parasite relation has markedly affected the microorganisms that became parasitic and contributed significantly to their phylogenetic differentiation.[124, 126] For the most part, such effects are inferred from existing kinds of microorganisms and the diseases they produce, but in the case of syphilis the relative mildness of the modern disease as contrasted with its severity in the early sixteenth century has been taken by some to be indicative of a reduced virulence of the spirochete for man.

If it be assumed that an important factor in the survival of the parasite is the extent to which it achieves a relatively innocuous but persisting infection in the host, from the microbial point of view the microorganism that produces a highly fatal disease may be said to have failed. It must be noted in this connection, however, that the causative agents of highly fatal diseases of man are not necessarily evolutionary failures; the high fatality of certain rickettsial diseases such as spotted fever, for example, is a consequence of adaptation to a host other than man, in this case the arthropod which is the vector from the anthropocentric point of view. To the microorganism the arthropod is the host, and human infection is an inconsequential episode in its life history.

The adaptation of the microorganism to the tissues of the host may be accompanied by an increasing dependence upon the host. Varying degrees of dependence are clearly evident as the generally fastidious nutritive requirements of the parasitic microorganisms and by host, and even tissue, specificities. In the first instance most pathogenic bacteria that occur within the host tissue proper require a relatively rich culture medium for growth and are often significantly altered by cultivation *in vitro*, as shown by loss of ability to invade the host, less exacting nutritive requirements, etc. as described earlier. Still others, such as the syphilis spirochete and the leprosy bacillus, have not been cultivated *in vitro*, and the rickettsiae and viruses are distinguished by their requirement for a living cell substrate for growth and a lack of other than token independent metabolic activity at the most. While the viruses are thought by some to be extremely primitive microorganisms, it is generally believed that they are degenerated forms occurring in continuous series from the rickettsiae, which serve as a connecting link to the bacteria, through the agents of the psittacosis-lymphogranuloma group, to the larger viruses of the pox group, and culminating in the viruses consisting only of nucleoprotein.

In the second instance, a microorganism may be well adapted to some kinds of tissue but not to others. For example, the meningococcus occurs widely in the upper respiratory tract of man and is restricted to the human host, but when it invades the central nervous system, it produces a highly fatal disease. Similarly, infection with poliomyelitis virus is very common and confined to man, but only in the rare individual does it damage the host to give symptoms of the

disease. The green streptococci are universally present in the mouth and upper respiratory tract of man, and their presence is regarded as "normal," but when they infect the valves of the heart, the subacute bacterial endocarditis produced is a highly fatal, though rare, disease. In effect, infection may be widespread but not overtly apparent, with actual disease occurring only rarely. Such a microorganism is a well-adapted parasite and is subjected only rarely to the unfortunate happenstance of causing fatal disease.

The extent to which the microbial variations are significant factors in a general pattern of evolutionary differentiation and specialization of microorganisms is a matter of speculation, but no more so than the role of classic genetics in the evolution of higher forms. At the same time, such variations are an important factor in the finer distinctions between microorganisms. Microbial phylogeny may be inferred with respect to broad tendencies and groups of microorganisms but apparently does not penetrate to the levels ascribed to microbial genus and species. For instance, when the antigenic structure of bacilli of the Salmonella group began to be precisely defined, a number of "trees" purporting to show a phylogenetic relationship among the antigenically different types were constructed. This is no longer of any interest because it has become clear that such types may be shown to occur by transduction and recombination, and it is highly probable that antigenic types are produced in nature in this way, particularly by phage-mediated transduction. From the phylogenetic point of view, the fertility groups, rather than subdivisions of them, are the important element, but as yet these are poorly understood.

REFERENCES

1. Abraham, E. P.: The development of drug resistance in microorganisms. Society for General Microbiology, Symposium No. 3, pp. 201–234. Cambridge University Press, 1953.
2. Adelberg, E. A. (ed.): Papers on Bacterial Genetics. Little, Brown and Company, Boston. 1960.
3. Anfinsen, C. B.: Genetic control of protein structure in bacteriophages. Fed. Proc., 1961, **20:**634–640.
4. Anker, H. S.: On the geogenous evolution of self-reproducing systems and macromolecules. Perspect. Biol. Med., 1961, **5:**86–88.
5. Armitage, P.: The statistical theory of bacterial populations subject to mutation. J. Roy. Statistical Soc., Ser. B, 1952, **14:**1–33.
6. Armitage, P.: Statistical concepts in the theory of bacterial mutation. J. Hyg., 1953, **51:**162 184.
7. Austrian, R.: Bacterial transformation reactions. Bact. Rev., 1952, **16:**31–50.
8. Barber, M.: Antibiotic-resistant staphylococcal variants. Society for General Microbiology, Symposium No. 3, pp. 235–252. Cambridge University Press, 1953.
9. Barber, M., et al.: Reversal of antibiotic resistance in hospital staphylococcal infection. Brit. Med. J., 1960, **i:**11–17.
10. Barksdale, L., Garmise, L., and Rivera, R.: Toxinogeny in *Corynebacterium diphtheriae*. J. Bact., 1961, **81:**527–540.
11. Baron, S., and Jensen, K. E.: Evidence for genetic interactions between non-infectious and infectious influenza A viruses. J. Exptl. Med., 1955, **102:**677–697.
12. Baron, L. S., Spilman, W. M., and Carey, W. F.: Hybridization of Salmonella species by mating with *Escherichia coli*. Science, 1959, **130:** 566–567.
13. Baron, L. S., Spilman, W. M., and Carey, W. F.: Diploid heterozygous hybrids from matings between *Escherichia coli* and *Salmonella typhosa*. J. Exptl. Med., 1960, **112:** 361–372.
14. Beale, G. H., and Wilkinson, J. F.: Antigenic variation in unicellular organisms. Ann. Rev. Microbiol., 1961, **15:**263–296.
15. Blum, H. F.: On the origin and evolution of living machines. Amer. Scientist, 1961, **49:** 474–501.
16. Bracco, R. M., Krauss, M. R., Roe, A. S., and MacLeod, C. M.: Transformation reactions between pneumococcus and three strains of streptococci. J. Exptl. Med., 1957, **106:**247–259.
17. Braun, W.: Bacterial Genetics. W. B. Saunders Company, Philadelphia. 1953.
18. Brenner, S.: Physiological aspects of bacteriophage genetics. Adv. Virus Res., 1959, **6:**137–158.
19. Bresch, C.: Recombination in bacteriophage. Ann. Rev. Microbiol., 1959, **13:**313–334.
20. Burnet, F. M., and Lind, P. E.: Recombination between the influenza virus strains WSE and CAM. Australian J. Exptl. Biol. Med. Sci., 1955, **33:**281–295.
21. Calvin, M.: Chemical evolution and the origin of life. Amer. Scientist, 1956, **44:**248–263.
22. Calvin, M.: Evolution of enzymes and photosynthetic apparatus. Science, 1959, **130:**1170–1174.
23. Campbell, A.: Synchronization of cell division. Bact. Rev., 1957, **21:**263–272.
24. Carpenter, C. M.: Gonococcal resistance to penicillin in the light of recent literature. Bull. W.H.O., 1961, **24:**321–326.
25. Catcheside, D. G.: Relation of genotype to enzyme content. Society for General Microbiol-

ogy, Symposium No. 10, pp. 181–207. Cambridge University Press, 1960.
26. Cavalli-Sforza, L. L.: Bacterial genetics. Ann. Rev. Microbiol., 1957, **11:**391–418.
27. Clowes, R. C.: Fine genetic structure as revealed by transduction. Society for General Microbiology, Symposium No. 10, pp. 92–114. Cambridge University Press, 1960.
28. Cohen, G. N., and Monod, J.: Bacterial permeases. Bact. Rev., 1957, **21:**169–194.
29. Cohen, S. S.: Virus-induced acquisition of metabolic function. Fed. Proc., 1961, **20:**641–649.
30. Cohn, M.: Contributions of studies on the β-galactosidase of *Escherichia coli* to our understanding of enzyme synthesis. Bact. Rev., 1957, **21:**140–168.
31. Cohn, M., and Monod, J.: Specific inhibition and induction of enzyme biosynthesis. Society for General Microbiology, Symposium No. 3, pp. 132–149. Cambridge University Press, 1953.
32. Cohn, M. L., Kovitz, C., Oda, V., and Middlebrook, G.: Studies on isoniazid and tubercle bacilli: Growth requirements, catalase activities and pathogenic properties of isoniazid-resistant mutants. Amer. Rev. Tuberc., 1954, **70:**641–664.
33. Cole, L. J., and Braun, W.: The effect of ionic Mn and Mg on the variation of *Brucella abortus*. J. Bact., 1950, **60:**283–289.
34. Dean, A. C. R.: Chloramphenicol resistance of *Bact. lactis aerogenes* (*Aerobacter aerogenes*). II. Production of highly resistant strains in non-selective conditions. Proc. Roy. Soc., Ser. B, 1961, **153:**329–339.
35. Dean, A. C. R., and Hinshelwood, C. N.: Observations on bacterial adaptation. Society for General Microbiology, Symposium No. 3, pp. 21–45. Cambridge University Press, 1953.
36. Demerec, M.: Genetic basis of acquired drug resistance. Pub. Health Repts., 1955, **70:**817–821.
37. Demerec, M.: Complex loci in microorganisms. Ann. Rev. Microbiol., 1959, **13:**377–406.
38. Dienes, L., and Weinberger, H. J.: The L forms of bacteria. Bact. Rev., 1951, **15:**245–288.
39. Edney, M.: Variations in animal viruses. Ann. Rev. Microbiol., 1957, **11:**23–42.
40. English, A. R., McBride, T. J., and Huang, H. T.: Microbial resistance to penicillin as related to penicillinase or penicillin acylase activity. Proc. Soc. Exptl. Biol. Med., 1960, **104:**547–549.
41. Ephrussi-Taylor, H.: Current status of bacterial transformations. Adv. Virus Res., 1955, **3:**275–307.
42. Fenner, F.: Genetic studies with mammalian pox viruses. II. Recombination between two strains of vaccinia virus in single HeLa cells. Virology, 1959, **8:**499–507.
43. Fenner, F., and Comben, B. M.: Genetic studies with mammalian pox viruses. I. Demonstration of recombination between two strains of vaccinia virus. Virology, 1958, **6:**530–548.
44. Finland, M.: Changing patterns of resistance of certain common pathogenic bacteria to antimicrobial agents. New England J. Med., 1955, **252:**570–580.
45. Finland, M.: Emergence of antibiotic-resistant bacteria. New England J. Med., 1955, **253:**909–922.
46. Finland, M.: Emergence of antibiotic-resistant bacteria. New England J. Med., 1955, **253:**969–979.
47. Finland, M.: Emergence of antibiotic-resistant bacteria. New England J. Med., 1955, **253:**1019–1028.
48. Fisher, M. W.: The antagonism of tuberculostatic action of isoniazid by hemin. Amer. Rev. Tuberc., 1954, **69:**469–470.
49. Fox, S. W.: How did life begin? Science, 1960, **132:**200–208.
50. Fox, M. S., and Hotchkiss, R. D.: Initiation of bacterial transformation. Nature, 1957, **179:**1322–1325.
51. Fraser, K. B.: Features of the MEL × NWS recombination systems in influenza A virus. II. The formation of N-M, recombinant, strains of influenza A virus in the mouse brain. Virology, 1959, **9:**178–190.
52. Fraser, K. B.: Features of the MEL × NWS recombination systems in influenza A virus. IV. Increments of virulence during successive cycles of double infection with two strains of influenza A virus. Virology, 1959, **9:**202–214.
53. Gaffron, H.: The origin of life. Perspect. Biol. Med., 1960, **3:**163–212.
54. Giessing, H. C.: Increasing failure rate after using the same preparation and dosage of penicillin in the treatment of gonorrhoea. Brit. J. Venereal Dis., 1959, **35:**256–257.
55. Gillisen, G., and Gillisen, I. M.: Die Resistenzbildung bei Mikroorganismen. Ergeb. Mikrobiol. Immunitätsf., 1960, **33:**128–141.
56. Goodlow, R. J., Mika, L. A., and Braun, W.: The effect of metabolites upon growth and variation of *Brucella abortus*. J. Bact., 1950, **60:**291–300.
57. Gots, J. S., and Sevag, M. G.: Enzymatic studies on the mechanism of the resistance of pneumococcus to drugs. I. Studies of the dehydrogenase activities and interrelationships of pneumococci, susceptible and resistant to acriflavine, atabrine, optochin, propamidine and sulfonamides. J. Bact., 1948, **56:**709–722.
58. Groman, N. B.: Evidence for the active role of bacteriophage in the conversion of nontoxigenic *Corynebacterium diphtheriae* to toxin production. J. Bact., 1955, **69:**9–15.
59. Groman, N. B., and Eaton, M. J.: Genetic factors in *Corynebacterium diphtheriae* conversion. J. Bact., 1955, **70:**637–640.
60. Halvorson, H. O., and Spiegelman, S.: The inhibition of amino acid formation by amino acid analogues. J. Bact., 1952, **64:**207–221.
61. Hanafusa, T., Hanafusa, H., and Kamahora, J.: Transformation of ectromelia into vaccinia virus in tissue culture. Virology, 1959, **8:**525–527.
62. Hanafusa, H., Hanafusa, T., and Kamahora, J.: Transformations of myxoma into vaccinia or ectromelia virus in tissue culture. Nature, 1959, **184:**1152–1153.
63. Hartman, P. E., and Goodgal, S. H.: Bacterial genetics (with particular reference to genetic transfer). Ann. Rev. Microbiol., 1959, **13:**465–504.
64. Hayes, W.: The bacterial chromosome. Society for General Microbiology, Symposium No. 10, pp. 12–38. Cambridge University Press, 1960.
65. Henry, B. S.: Dissociation in the genus Brucella. J. Infect. Dis., 1933, **52:**374–402.
66. Hinshelwood, C. N.: The Chemical Kinetics

of the Bacterial Cell. Clarendon Press, Oxford. 1946.
67. Hirsch, H. A., and Finland, M.: Susceptibility of gonococci to antibiotics and sulfadiazine. A study of strains isolated at Boston City Hospital from cases of acute gonorrheal urethritis in males, August, 1958 to February, 1959. Amer. J. Med. Sci., 1960, **239:** 41–50.
68. Holtman, D. F.: The acquisition of heterophile antigen by *Eberthella typhosa* and *Salmonella paratyphi* during culture on artificial media. J. Immunol., 1939, **36:**405–412.
69. Holtman, D. F.: The acquisition of heterophile antigen from the guinea pig by *Eberthella typhosa* and *Salmonella paratyphi.* J. Immunol., 1939, **36:**413–424.
70. Hoskins, J. M.: Host-controlled variation in animal viruses. Society for General Microbiology, Symposium No. 9, pp. 122–141. Cambridge University Press, 1959.
71. Hotchkiss, R. D.: Bacterial transformation. J. Cell. Comp. Physiol., 1955, **45:** Suppl. 2, 1–22.
72. Hurst, G. K., and Gotlieb, T.: The experimental production of combination forms of virus. V. Alterations in the virulence of neurotropic influenza virus as a result of mixed infections. Virology, 1955, **1:**221–235.
73. Jacob, F., Schaeffer, P., and Wollman, E. L.: Episomic elements in bacteria. Society for General Microbiology, Symposium No. 10, pp. 67–91. Cambridge University Press, 1960.
74. Jacob, F., and Wollman, E. L.: Sexuality and the Genetics of Bacteria. Academic Press, New York. 1961.
75. Jawetz, E.: Antimicrobial chemotherapy. Ann. Rev. Microbiol., 1956, **10:**85–114.
76. Kilham, L.: The fibroma-myxoma transformation. Adv. Virus Res., 1960, **7:**103–129.
77. Kleineberger-Nobel, E.: Filterable forms of bacteria. Bact. Rev., 1951, **15:**77–103.
78. Kleineberger-Nobel, E.: Microorganisms of the pleuropneumonia group. Biol. Rev., 1954, **29:**154–184.
79. Kleineberger-Nobel, E.: L-forms of bacteria. In Gunsalus, I. C., and Stanier, R. Y. (eds.): The Bacteria, vol. I, pp. 361–386. Academic Press, New York. 1960.
80. Knox, R.: Haemin and isoniazide resistance of *Mycobacterium tuberculosis.* J. Gen. Microbiol., 1955, **12:**191–202.
81. Koprowski, H.: Importance of genetics of viruses in medical research. Amer. J. Human Genetics, 1959, **11:**335–351.
82. Kushner, D. J., and Quastel, J. H.: Factors underlying bacterial enzyme synthesis. Proc. Soc. Exptl. Biol. Med., 1953, **82:**388–392.
83. Lederberg, J.: Recombination mechanisms in bacteria. J. Cell. Comp. Physiol., 1955, **45:** Suppl. 2, 75–107.
84. Lederberg, J.: Genetic transduction. Amer. Scientist, 1956, **44:**264–280.
85. Lederberg, J.: Viruses, genes and cells. Bact. Rev., 1957, **21:**133–139.
86. Lederberg, J.: A view of genetics. Science, 1960, **131:**269–276.
87. Lederberg, J., and Lederberg, E. M.: Replica plating and indirect selection of bacterial mutants. J. Bact., 1952, **63:**399–406.
88. Lederberg, J., and St. Clair, J.: Protoplasts and L-type growth of *Escherichia coli.* J. Bact., 1958, **75:**143–160.
89. Lepper, M. H.: Microbial resistance to antibiotics. Ann. Intern. Med., 1955, **43:**299–315.
90. Lind, P. E., and Burnett, F. M.: Recombination between virulent and non-virulent strains of influenza virus. I. The significance of heterozygosis. Australian J. Exptl. Biol. Med. Sci., 1957, **35:**57–66.
91. Lind, P. E., and Burnett, F. M.: Recombination between neurotropic and non-neurotropic influenza virus strains. Australian J. Exptl. Biol. Med. Sci., 1954, **32:**437–452.
92. Litwin, J.: A simple method for cultivation of viruses and rickettsiae in the chorioallantoic ectoderm of the chick embryo by inoculation via the air sac. J. Infect. Dis., 1957, **101:**100–108.
93. Luria, S. E.: Recent advances in bacterial genetics. Bact. Rev., 1947, **11:**1–40.
94. Luria, S. E., Adams, J. N., and Ting, R. C.: Transduction of lactose-utilizing ability among strains of *E. coli* and *S. dysenteriae* and the properties of the transducing phage particles. Virology, 1960, **12:**348–390.
95. Luria, S. E., and Burrous, J. W.: Hybridization between *Escherichia coli* and Shigella. J. Bact., 1957, **74:**461–476.
96. Mandelstam, J.: The mass action theory of enzyme adaptation. Biochem. J., 1952, **51:** 674–681.
97. Middlebrook, G.: Isoniazide-resistance and catalase activity of tubercle bacilli: preliminary report. Amer. Rev. Tuberc., 1954, **69:** 471–472.
98. Miller, S. L., and Urey, H. C.: Organic compound synthesis on the primitive earth. Science, 1959, **130:**245–251.
99. Muller, H. J.: Genetic nucleic acid: Key material in the origin of life. Perspect. Biol. Med., 1961, **5:**1–23.
100. Oparin, A.: The Origin of Life on the Earth. Academic Press, New York. 3rd ed., 1957.
101. Padron, J. L., Smith, S. C., and Ramsey, H. H.: Chloramphenicol resistance in *Micrococcus pyogenes.* II. Intermediary metabolism of sensitive and resistant strains. Proc. Soc. Exptl. Biol. Med., 1954, **87:**477–478.
102. Page, L. A., Goodlow, R. J., and Braun, W.: The effect of threonine on population changes and virulence of *Salmonella typhimurium.* J. Bact., 1951, **62:**639–647.
103. Perry, B. T., Van Den Ende, M., and Burnett, F. M.: Recombination with two influenza B strains in the de-embryonated egg. Australian J. Exptl. Biol. Med. Sci., 1954, **32:**469–478.
104. Plough, H. H., and Grimm, M. R.: Reversal to penicillin sensitivity in a cysteine-requiring mutant of Salmonella. Science, 1949, **109:** 173–174.
105. Pollock, M. R.: Stages in enzyme adaptation. Society for General Microbiology, Symposium No. 3, pp. 150–183. Cambridge University Press, 1953.
106. Pollock, M. R.: Induced formation of enzymes. In Boyer, P. D., Lardy, H., and Myrbäck, K. (eds.): The Enzymes, vol. 1, pp. 619–680. Academic Press, New York. 2nd ed., 1959.
107. Pollock, M. R.: Drug resistance and mechanisms for its development. Brit. Med. J., 1960, **ii:**16–22.
108. Ravin, A. W.: Bacterial genetics. Ann. Rev. Microbiol., 1958, **12:**309–364.
109. Report: Emergence of bacterial resistance in pulmonary tuberculosis under treatment with isoniazide, streptomycin plus PAS and strep-

tomycin plus isoniazide. Lancet, 1953, **ii:** 217–223.
110. Rosanoff, E. I., and Sevag, M. G.: Alternate metabolic pathways in streptomycin sensitive and resistant strains of *Escherichia coli*. Antibiotics and Chemotherapy, 1953, **3:**495–504.
111. Salser, W.: Non-genetic biological information mechanisms. Perspect. Biol. Med., 1961, **4:** 177–198.
112. Saz, A. K., and Martinez, L. M.: Enzymatic basis of resistance to Aureomycin. II. Inhibition of electron transport in *Escherichia coli* by Aureomycin. J. Biol. Chem., 1958, **233:** 1020–1022.
113. Schneider, H., Formal, S. B., and Baron, L. S.: Experimental genetic recombination in vivo between *Escherichia coli* and *Salmonella typhimurium*. J. Exptl. Med., 1961, **114:**141–148.
114. Schnitzer, R. J., and Grunberg, E.: Drug Resistance of Microorganisms. Academic Press, New York. 1957.
115. Sevag, M. G.: Enzyme problems in relation to chemotherapy "adaptations," mutations, resistance and immunity. Adv. Enzymol., 1946, **6:**33–121.
116. Sevag, M. G., and Gots, J. S.: Enzymatic studies on the mechanism of the resistance of pneumococcus to drugs. II. The inhibition of dehydrogenase activities by drugs: antagonistic effects of riboflavin to inhibitions. J. Bact., 1948, **56:**723–735.
117. Sevag, M. G., and Gots, J. S.: Enzymatic studies on the mechanism of the resistance of pneumococcus to drugs. III. Experimental results indicating alteration in enzyme proteins associated with the development of resistance to drugs. J. Bact., 1948, **56:**737–748.
118. Sevag, M. G., and Rosanoff, E. I.: Mechanism of the development of resistance to streptomycin. I. Origin of resistant strains. J. Bact., 1952, **63:**243–251.
119. Sneath, P. H., and Lederberg, J.: Inhibition by periodate of mating in *Escherichia coli* K-12. Proc. Nat. Acad. Sci., 1961, **47:**86–90.
120. Spiegelman, S., and Halvorson, H. O.: The nature of the precursor in the induced synthesis of enzymes. Society for General Microbiology, Symposium No. 3, pp. 98–131. Cambridge University Press, 1953.
121. Spiegelman, S., and Landman, O. E.: Genetics of microorganisms. Ann. Rev. Microbiol., 1954, **8:**181–236.
122. Sprunt, K., Redman, W. M., and Alexander, H. E.: Combination of antigenic traits of type 1 and type 2 poliovirus. J. Immunol., 1959, **82:**232–240.
123. Stanier, R. Y.: Enzymatic adaptation in bacteria. Ann. Rev, Microbiol., 1951, **5:**35–56.
124. Stanier, R. Y.: Adaptation, evolutionary and physiological: or Darwinism among the microorganisms. Society for General Microbiology, Symposium No. 3, pp. 1–20. Cambridge University Press, 1953.
125. Stent, G.: Mating in the reproduction of bacterial viruses. Adv. Virus Res., 1958, **5:**95–150.
126. Stocker, B. A. D.: Bacterial genetics and infectious disease. Amer. J. Human Genetics, 1959, **11:**354–365.
127. Strauss, B. S.: An Outline of Chemical Genetics. W. B. Saunders Company, Philadelphia. 1960.
128. Symposium: Adaptation in Microorganisms. Society for General Microbiology, Symposium No. 3. Cambridge University Press, 1953.
129. Symposium: First International Symposium on the Origin of Life on Earth (Moscow, 1957). Pergamon Press, New York. 1959.
130. Symposium: Virus Growth and Variation. Society for General Microbiology, Symposium No. 9. Cambridge University Press, 1959.
131. Symposium: Microbial Genetics. Society for General Microbiology, Symposium No. 10. Cambridge University Press, 1960.
132. Tulasne, R., Minck, R., Kirn, A., and Krembel, J.: Délimitation de la notion de formes L des bactéries: protoplasts, spheroplastes et formes L. Ann. Inst. Pasteur, 1960, **99:**859–874.
133. Turk, D. C.: Shigellosis and sulphonamides. Lancet, 1960, **ii:**460–461.
134. Weibull, C.: Chemical analyses elucidating the structure of bacterial L forms. Acta. Path. Microbiol. Scand., 1958, **42:**324–332.
135. Weiss, E.: The extracellular development of agents of the psittacosis-lymphomagranuloma group (Chlamydoboaceae). J. Infect. Dis., 1949, **84:**125–149.
136. Willcox, R. R.: Treatment problems of gonorrhoea. Bull. W.H.O. 1961, **24:**307–319.
137. Woodroofe, G. M., and Fenner, F.: Genetic studies with mammalian pox viruses. IV. Hybridization between several different poxviruses. Virology, 1960, **12:**272–282.
138. Woof, J. B., and Hinshelwood, C. N.: Chloramphenicol resistance of *Bact. lactis aerogenes* (*Aerobacter aerogenes*). I. Adaptive and lethal processes in liquid media and on agar plates. Proc. Roy. Soc., Ser. B, 1961, **153:** 321–328.
139. Yanofsky, C., and St. Lawrence, P.: Gene action. Ann. Rev. Microbiol., 1960, **14:**311–340.
140. Yudkin, J.: Enzyme variation in microorganisms. Biol. Rev., 1938, **13:**93–106.
141. Zelle, M. R.: Genetics of microorganisms. Ann. Rev. Microbiol., 1955, **9:**45–96.
142. Zinder, N. D.: Bacterial transduction. J. Cell. Comp. Physiol., 1955, **45:** Suppl. 2, 23–49.
143. Zinder, N. D.: Hybrids of Escherichia and Salmonella. Science, 1960, **131:**813–815.
144. Zinder, N. D.: Sexuality and mating in Salmonella. Science, 1960, **131:**924–926.

Chapter 8

THE TAXONOMY OF MICROORGANISMS

Of the microorganisms, the fungi are clearly plants and the animal parasites are unicellular and multicellular animals, and both groups are formally classified on the conventional morphological basis as described elsewhere (Chaps. 32 and 34). The bacteria and viruses occupy an intermediate position between the plant and animal kingdoms. They are linked to the fungi through the actinomyces and plant-like bacteria, especially the mycobacteria (tubercle and related bacilli) and the corynebacteria (diphtheria and diphtheroid bacilli). Their relation to the animal kingdom through the protozoa is less clear, though the spirochetes are considered by many to be more animal-like than other bacteria. The rickettsiae and viruses are perhaps best regarded as offshoots of the bacteria and related to the plants and animals proper only through the bacteria.

Since the bacteria have properties of both plants and animals, at one time the question of whether they should be allocated to one kingdom or the other was an active one. An alternative proposal that gained some support, but not acceptance, was the creation of a new third kingdom, the Protista, for these forms. In time it became apparent that this question is irrelevant in the sense that an unequivocal answer to it serves no useful purpose, and the whole matter has been largely dropped from active consideration. Relatively early, however, the opinion prevailed that, in spite of the occurrence of both plantlike and animal-like properties among the bacteria, in total they resemble plants more than animals, and they were formally classified with the plants under Thallophyta as Schizomycetes, or fission fungi. The postulated relationship to other plant phyla is indicated in the accompanying outline. At the same time, the prominence of comparative physiology in the characterization and differentiation of the bacteria and the animal-like nature of their physiological processes leads many bacteriologists to think of them casually as animals and refer to them as "experimental animals." There is, of course, little or no basis for classification of the rickettsiae and viruses as plants, or animals either, for that matter.

Although the interrelationships of the organisms included under Schizomycetes present problems which are, in many essentials, new to the taxonomist, certain "natural" groups are apparent. Of these the most obvious is that based on morphology, with primary division into spherical, rod-shaped, spiral, and filamentous forms, and subdivision on the basis of spore formation, presence and location of flagella, and staining reactions to the Gram and acid-fast stains. The earlier classifications, the better known of which are those of Migula

Phylum I. *Thallophyta,* plants without distinction of root, stem and branch
Subphylum 1—the algae
Subphylum 2—the fungi—thallophytes lacking chlorophyll
Class I. *Schizomycetes*—the bacteria
Class II. *Myxomycetes*—the slime molds
Class III. *Phycomycetes*—the algae-like fungi
Class IV. *Ascomycetes*—the fungi which form ascospores
Class V. *Basidiomycetes*—the fungi which form basidiospores
Phylum II. *Bryophyta*—the mosses
Phylum III. *Pteridophyta*—the ferns
Phylum IV. *Spermatophyta*—the seed-bearing plants

and of Lehmann and Neumann, were made on this basis. These classifications exerted a strong influence on bacterial taxonomy and resulted in the naming of a great many species of bacteria, many of which persist in current classifications.

Morphology is, however, not a sufficient basis for the separation of bacterial species, for many morphologically similar organisms may be quite different in other respects. Physiological differences, generally readily determinable in the laboratory, have been widely used. In fact, one of the early classifications, that of Orla-Jensen, depended upon nutritional differences for the primary subdivision into three main groups:

(1) The autotrophic bacteria which, like the green plants, require neither organic carbon nor organic nitrogen compounds;
(2) Bacteria which require organic carbon compounds but can dispense with organic nitrogen, using ammonium salts or other inorganic compounds of nitrogen;
(3) Bacteria which, like higher animals, require both organic carbon and organic nitrogen compounds.

A number of workers, especially Kluyver and van Niel,[7] are of the opinion that comparative physiology should constitute the primary basis of separation, rather than being subordinate to morphology.

Beyond such primary subdivisions, classification becomes increasingly difficult, and it is quite clear that this is so because not nearly enough is known of the phylogenetic relationships of the bacteria to one another for a detailed classification with definition of genera and species. This question has been discussed in some detail by van Niel,[8] who shows that as yet any classification can be little more than a key, and species only "form" species, that is to say, no more than convenient handles.

The fundamental difficulty in preparing a classification rather than a differential key for the bacteria and viruses is a general uncertainty as to the significance or triviality of the differential characters used. Inevitably, a sound taxonomy will need to be based on microbial genetics and fertility systems, the latter in the broad sense to include the transduction reactions as well as sexual recombination. While these mechanisms are by now well established, they have not been sufficiently widely applied to know, for example, how common or uncommon asexual strains of microorganisms not amenable to genetic transfer may be. Still other approaches include the occurrence of key cellular constituents determinable by biochemical analysis; there is, for instance, some biochemical basis for Gram stain differentiation, evidence suggesting that content of amino acids and sugars, as determined by chromatography, and the purine and pyrimidine base ratios in microbial DNA show some correlation with genera and species, etc.

While the variability of microorganisms is significant to classification, it is not, as might be supposed, a deterrent to differentiation and characterization by the usual physiological and immunological methods. Instability becomes evident when the environment is varied, but ordinarily microorganisms isolated in the same way retain their individuality for purposes of identification, or, if variation is common, as the O-D variation of influenza virus on primary isolation in the allantoic cavity of the embryonated hen's egg, it occurs with some

regularity and is taken into consideration. Aberrant forms occur from time to time, of course, but in general an identification key is both practical and useful even though it may fail to represent genetic or other relationships.

Bacterial classification has evolved through some sixty odd schemes to perhaps half a dozen current today. The criteria made use of are, in the order of approximate fineness of distinction made:

(1) morphology—both gross and microscopic
(2) physiology—biochemical properties
(3) pathogenicity—of the disease-producing bacteria
(4) immunology—the antigenic structure of the bacterial cell

Of the current classifications that of the Bergey Manual is by far the most detailed, including in the sixth edition a classification of the rickettsiae and viruses, and is the only one that need be considered here. It developed from the earlier classification of Chester and the work of a committee of the Society of American Bacteriologists, but is not, as is sometimes supposed, the official expression of the views of the Society. The Bergey classification has gained ascendancy in this country in recent years, though it is seldom used outside the United States. This classification is a key rather than a taxonomy based on phylogenetic and genetic relationships, and the difficulties inherent in the preparation of a work of this nature are apparent in the frequency with which bacteria are encountered which do not fit into more than the larger divisions of the key. An abridged and workable general key based on this classification has been prepared by Skerman.[10]

An abridged outline of the Bergey (1957) classification is given in the accompanying diagrammatic form. The variations which appear have been retained to keep the nomenclature from differing too widely from practice in other than current American literature. For example, the name *Vibrio comma* for the cholera vibrio is not used outside the Bergey classification; over 98 per cent of the papers published on this bacterium in the past 20 years are not American and consistently use the name *Vibrio cholerae*. Abridged keys for the differentiation of the better-known species of bacteria are included in subsequent chapters.

Aside from a few abortive attempts, the viruses have thus far eluded the systematists, illustrating the inability of classic taxonomy to cope with the macromolecular basis of modern microbiology. Viral nomenclature illustrates too the virtues of usage. When group designations have become a practical necessity, appropriate and useful terms tend to be coined by active workers; these include various contractions such as poliovirus, poxvirus, arbor virus, for arthropod-borne virus, and ECHO virus, for enteric cytopathogenic human orphan virus, together with certain other designations such as myxovirus for the viruses of the influenza group. Otherwise viruses are named informally after the disease they cause. A formal nomenclature, as on the Linnean model, is obviously premature if indeed applicable.[1]

Nomenclature. Neither the botanical nor zoological codes of nomenclature have been satisfactorily applicable to the bacteria and viruses, and an official International Code of Nomenclature of Bacteria and Viruses has been, and continues to be, developed by committees appointed by the several International Congresses of Microbiology. In general a bacterium has a generic name, always written with a capital letter, which may or may not be descriptive (for example, *Bacillus*—a small rod; or *Pasteurella*—in honor of Pasteur) and a specific name which may be an adjective (*albus*—white) or a noun indicating possession (*Clostridium welchii*—Welch's clostridium) or a noun in apposition (*Bacillus radicicola*—the root-dweller bacillus). The practice of indicating the author of the name, as in *Bacillus subtilis* Cohn, is not as common in bacteriology as in zoology or botany. The use of trinomial and quadrinomial names such as *Granulobacillus saccharobutyricus mobilis nonliquefaciens* is obviously highly undesirable. Trinomials are occasionally

useful in the designation of varieties of subspecies. Common names are, of course, used; terms such as Friedländer's bacillus and the typhoid bacillus are frequently encountered.

Genera. Perhaps one of the greatest difficulties the bacterial taxonomist labors under is the paucity of genera. The question of what degree of difference shall be judged sufficient to establish new genera is one to which there is as yet no satisfactory answer. At the present time considerable confusion and lack of uniformity still exist. In a number of instances new generic names have obtained wide currency among bacteriologists in many parts of the world, partly, doubtless, because they are applied to fairly distinct groups of microorganisms and have genuine classificatory value. Such are Brucella for the bacilli of undulant fever of man and contagious abortion of cattle, Salmonella for the paratyphoid bacilli, and Pasteurella for the bacilli of hemorrhagic septicemia in domestic animals and the bacilli of plague and tularemia; the name Shigella for the dysentery bacilli has gained a considerable degree of recognition. Nevertheless, the status of genera of bacteria, and viruses especially, is questionable in that they do not have the status of botanical and zoological genera based on character or gene frequencies and capacity for hybridization. The cross-fertility shown by recombination between Escherichia and Shigella and Salmonella referred to earlier (Chap. 7) is a clear indication of the uncertainties in definitions of bacterial genera.

A practical consequence of such uncertainty has been a degree of instability in generic names with resulting confusion. Staphylococcus becomes Micrococcus, and then returns to Staphylococcus, and the glanders bacillus leaps nimbly from Actinobacillus to Pfeifferella to Malleomyces, and back again to Actinobacillus, Hemophilus (pertussis) alternates with Brucella and then becomes Bordetella, and the tularemia microorganism perhaps should be Brucella rather than Pasteurella.

Species.[9, 14] The differentiation of species is made for the most part on a physiological basis. A single character is hardly sufficient, particularly in view of the facility with which variation occurs. The use of a number of such characters is the rule, but difficulties are frequently encountered when intermediate forms occur. This is common among the enteric bacilli, possibly because these are better known than many groups of bacteria. For example, among the coliform bacilli two commonly encountered forms which are little more than variants are separated into different species, *Bacterium coli* and *Bact. aerogenes,* by some workers, and into different genera, *Escherichia coli* and *Aerobacter aerogenes,* in the Bergey schema. Further, some formal designation for the paracolon bacilli has been urgently needed, but the proposed creation of a new genus Paracolobactrum is anomalous in that there is no corresponding genus Colobactrum which the name implies that it resembles. The divergence of views, which gives rise to such unsatisfactory situations, is the inevitable consequence of attempting to build a taxonomic structure, instead of a less ambitious working key, on an inadequate foundation of knowledge of the bacteria.

Types.[3] Immunological differentiation is generally regarded as not of species status though it may sometimes coincide with species differentiation on a physiological basis. Conversely, minor cross-reactions between species are not indicative of species identity. There are some exceptions to the former, however, and many workers divide the genus Salmonella into species on the basis of antigenic structure. Immunological differences are of very considerable value, particularly for epidemiological purposes, and are usually the basis of distinction of types within a species. Thus the streptococci are divided into groups, designated A, B, C, etc., on the basis of one kind of antigen, and into Arabic-numbered types within and across species by another. The pneumococci are similarly divided into numbered types on the basis of capsular antigen. *Clos-*

Classification of Microorganisms According to Bergey (1957)

ORDER	FAMILY	TRIBE	GENUS	
Pseudomonadales (suborder Pseudomonadineae)	Nitrobacteraceae		Nitrosomonas, Nitrosococcus, Nitrobacter	the nitrifying bacteria
	Methanomonadaceae		Methanomonas	methane bacteria
			Hydrogenomonas	the hydrogen bacteria
	Thiobacteriaceae		Thiospira	sulfur vibrio
			Thiobacillus	certain of the sulfur bacteria
	Pseudomonadaceae		Pseudomonas	Ps. aeruginosa *et al.*
			Acetobacter	acetic acid bacteria
	Spirillaceae		Vibrio	cholera, paracholera, and non-cholera vibrios
			Cellvibrio	cellulose oxidizing vibrios
			Spirillum	Sp. minus (rat-bite fever) and saprophytic species
Chlamydobacteriales				filamentous saprophytic bacteria, including sheathed iron bacteria
Eubacteriales	Azotobacteraceae		Azotobacter	non-symbiotic nitrogen-fixing bacteria
	Rhizobiaceae		Rhizobium	symbiotic nitrogen-fixing bacteria
			Agrobacterium	plant pathogens and saprophytes
			Chromobacterium	certain pigmented saprophytic bacteria
	Achromobacteraceae		Alcaligenes	Alc. fecalis and related forms
			Achromobacter	non-pigmented soil and water bacteria
			Flavobacterium	pigmented soil and water bacteria
	Enterobacteriaceae	Escherichieae	Escherichia, Aerobacter	coliform bacteria
			Klebsiella	Friedländer's bacillus
		Erwinieae	Erwinia	plant pathogens
		Serratieae	Serratia	Serratia marcescens (Chromobacter prodigiosum) and related forms
		Proteeae	Proteus	Pr. vulgaris and related forms
		Salmonelleae	Salmonella	typhoid and paratyphoid bacilli
			Shigella	dysentery bacilli
	Brucellaceae		Pasteurella	plague and hemorrhagic septicemia bacilli
			Brucella	bacilli of undulant fever and contagious abortion
			Haemophilus	influenza bacillus, pertussis bacillus, chancroid bacilli
			Actinobacillus	actinobacillosis, glanders bacillus

	Bacteroidaceae		Bacteroides	non-sporulating obligate anaerobes
	Micrococcaceae		Staphylococcus	the staphylococci
			Gaffkya	M. tetragenus and related bacteria
			Sarcina	S. lutea and other cocci
	Neisseriaceae		Neisseria	gonococcus, meningococcus, etc.
			Veillonella	certain anaerobic cocci
	Lactobacillaceae	Streptococceae	Diplococcus	pneumococcus and related forms
			Streptococcus	the streptococci
			Leuconostoc	saprophytic cocci
		Lactobacilleae	Lactobacillus	lactic acid bacteria
	Propionibacteriaceae		Propionibacterium	propionic acid bacteria
	Corynebacteriaceae		Corynebacterium	diphtheria and diphtheroid bacilli
			Listeria	L. monocytogenes
			Erysipelothrix	bacterium of erysipeloid and swine erysipelas
	Bacillaceae		Bacillus	aerobic, spore-forming bacilli such as B. anthracis, B. subtilis, etc.
			Clostridium	obligate anaerobic, spore-forming bacilli, including bacilli of tetanus, gaseous gangrene, and botulinus bacillus
Actinomycetales	Mycobacteriaceae		Mycobacterium	tubercle bacilli, leprosy bacilli, saprophytic forms
	Actinomycetaceae		Nocardia	aerobic, sometimes acid-fast actinomycetes
			Actinomyces	anaerobic actinomyces of actinomycosis
	Streptomycetaceae		Streptomyces	soil forms, often producing antibiotics
Spirochaetales	Spirochaetaceae		Spirochaeta	
			Saprospira	saprophytic water forms
			Cristispira	parasite of molluscs
	Treponemataceae		Borrelia	relapsing fever spirochetes
			Treponema	spirochetes of syphilis and yaws
			Leptospira	spirochetes of infectious jaundice and field fever
Mycoplasmatales	Mycoplasmataceae		Mycoplasma	pleuropneumonia-like organisms (PPLO)
Rickettsiales	Rickettsiaceae	Rickettsieae	Rickettsia	typhus fever, spotted fever, etc.
			Coxiella	Q fever
		Ehrlichieae	Cowdria	heartwater disease of cattle
	Bartonellaceae		Bartonella	bartonellosis of man
			Haemobartonella	bartonellosis of dogs, rodents, and cattle
Virales				viruses, infecting bacteria, plants and animals

tridium botulinum is separated into types, A, B, C, etc., on the basis of the immunological specificity of the toxin, and these have no relation to the immunological character of the cell substance. There is, then, no general practice with respect to type differentiation. Physiological differences within species are usually not used to differentiate types, and the biochemical reaction is simply given as variable.

REFERENCES

1. Andrewes, C. H., and Sneath, P. H. A.: The species concept among viruses. Nature, 1958, **182:**12–14.
2. Buchanan, R. E.: Taxonomy. Ann. Rev. Microbiol., 1955, **9:**1–20.
3. Buchanan, R. E.: Microbial taxa of intraspecific and infrasubspecific rank. Internat. Bull. Bact. Nomencl. Taxonom., 1958, **8:**97–109.
4. Buchanan, R. E.: Microbiological literacy. Bact. Rev., 1958, **22:**204–215.
5. Breed, R. S., Murray, E. G. D., and Smith, N. R.: Bergey's Manual of Determinative Bacteriology. Williams and Wilkins, Baltimore. 7th ed., 1957.
6. International Committee on Bacteriological Nomenclature. International Code of Nomenclature of Bacteria and Viruses. Iowa State College Press, Ames, Iowa. 1958.
7. Kluyver, A. J., and van Niel, C. B.: Prospects for a natural system of classification of bacteria. Cent. f. Bakt. u. Parasitenk., Abt. II, 1936, **94:**369–403.
8. van Niel, C. B.: The classification and natural relationships of bacteria. Cold Spring Harbor Symposia Quant. Biol., 1946, **11:**285–301.
9. Ravin, A. W.: The origin of bacterial species. Bact. Rev., 1960, **24:**201–220.
10. Skerman, V. B. D.: Guide to the Identification of the Genera of Bacteria. Williams and Wilkins, Baltimore. 1959.
11. Sneath, P. H. A.: The application of computers to taxonomy. J. Gen. Microbiol., 1957, **17:** 201–206.
12. Sneath, P. H. A.: Some thoughts on bacterial classification. J. Gen. Microbiol., 1957, **17:** 184–200.
13. Sneath, P. H. A., and Cowan, S. T.: An electrotaxonomic survey of bacteria. J. Gen. Microbiol., 1958, **19:**551–565.
14. Société Francaise de Microbiologie: Colloque annuel. La notion d'espèce bactérienne à la lumière des découvertes récentes. Ann. Inst. Pasteur, 1958, **94:**137–223.
15. Symposium: The principles of microbial classification. J. Gen. Microbiol., 1955, **12:**314–386.
16. Symposium: Microbial Classification. Society for General Microbiology, Symposium No. 12. Cambridge University Press, 1962.

Chapter 9

THE PATHOGENIC MICROORGANISMS AND DISEASE

Infectious disease may be regarded as a by-product, and not an inevitable one, of the host-parasite relationship. While, as suggested earlier, the initiation of the career of a parasitic microorganism may be based on its ability to produce sufficient damage to the host to facilitate the development of foci of infection, pathogenicity is incidental to the parasite and has significance only as it relates to the establishment of an ecological niche and to the survival of the microorganisms.

The etiological relation of microorganisms to infectious disease was suspected by some not long after their discovery by Leeuwenhoek and, in a sense, found support in Jenner's vaccination studies and those of others, especially the classic work of Snow on the transmission of cholera, in which a *contagium vivum* was implied. It was not until past the middle of the nineteenth century that, as described elsewhere (Chap. 1), this belief began to crystalize into knowledge through parallel developments such as the transmission of anthrax by infected blood by Brauell in 1867 and the recognition of the resemblance of fermentation processes to sepsis to provide the basis of Lister's antiseptic surgery, culminating in Koch's demonstration of the microbial etiology of anthrax. Through exploitation of his pure culture techniques in application to a wide variety of diseases, the etiology of many was resolved to displace the Hippocratic doctrine of imbalance of the four bodily humors, blood, phlegm, yellow bile, and black bile, as the basis of all disease.

THE SPECIFIC MICROBIAL ETIOLOGY OF INFECTIOUS DISEASE

It is one thing to suspect, or even have some evidence of, the ability of microorganisms to produce disease and quite another to develop unequivocal proof of a specific etiological relation between a given microorganism and a given disease.

Koch's Postulates. Such proof was first offered by Koch in the form of a logical chain of experimental evidence, usually known as Koch's postulates, although there is no evidence that they were so formulated by Koch, and good reason to believe that they originated with Koch's professor, Cohn. However

this may be, there are four of these; a fifth was suggested at one time but it is usually not generally applicable.

The first postulate. The first of these is the observed occurrence of the microorganism in association with the disease. This is relatively simple in some diseases in which the causative microorganism is found in pure culture in some kinds of specimens, *viz.*, the typhoid bacillus in the blood or the plague bacillus in fluid aspirated from the infected lymph nodes in the bubonic form of the disease. In others, however, notably infections of the respiratory and intestinal tracts, the causative microorganism occurs admixed with many other, often similar, forms and needs to be sorted out.

The requirement for such preliminary observation is self-evident in that if an association were not observed there would be no reason to suspect that it might be an etiological relation requiring further investigation. At the same time, it is necessary to emphasize the first postulate for a number of reasons. First, closely similar, even indistinguishable, clinical syndromes may have quite different etiology. For instance, it is not possible to distinguish among certain diarrheal diseases of varied etiology on clinical grounds alone, *e.g.*, acute bacillary dysentery and Asiatic cholera occurring, as often is the case, in debilitated persons; a variety of bacteria may be the cause of urinary tract disease; and the still unresolved problem of the etiology of the so-called common cold is largely a consequence of a wide variety of etiological agents which give rise to substantially the same symptoms.

Second, the term "observation" must be taken broadly. In the case of pathogenic bacteria there may be no morphological distinction between the pathogen and related nonpathogens, *e.g.*, bacteria such as the diphtheria and diphtheroid bacilli and the pathogenic enteric bacilli and nonpathogenic coliforms are morphologically indistinguishable. Further, when the infectious agent is too small to be resolved in the light microscope, as is the case with the viruses, it cannot be directly observed. Thus the first postulate melts into the second in that characterization and differentiation of the microorganism in isolated form are often, in fact usually, required to establish its presence in association with the disease.

Finally, the first postulate has been taken to imply not only presence of the microorganism in association with the disease, but also the corollary that it is absent in the absence of disease. Quite aside from the occurrence of similar microorganisms in the same specimen, only one of which may be related to the disease, the pathogenic microorganisms may be present in individuals who have no symptoms of the disease, *viz.*, inapparent casual infections, carriers, and other forms of asymptomatic infection. It was the presence of diphtheria bacilli in normal as well as diseased individuals that led Klebs, who discovered this microorganism, to question its etiological relation to the disease.

So, in practice, it is necessary to qualify the dogmatic first postulate in a number of ways, *viz.*, observation must be stretched to include, for example, the cytopathology of tissue culture inoculated with infectious material, an observed association in at least a majority of cases of the disease, and regard of the microorganism as suspect even though it may be found in healthy persons. Such association is by no means proof of an etiological relation, and further evidence is required.

The second postulate. The second postulate is that the microorganism must be isolated and grown in pure culture. This is clearly essential to effect a separation of the causative agent from other microorganisms when they are present and, as indicated above, is often an integral part of the first postulate for purposes of characterization of the agent. It is usually possible to isolate and grow in pure culture pathogenic bacteria, the practical difficulty being the requirement for an appropriate culture medium, but occasionally this cannot be done. The leprosy bacillus, regularly found in enormous numbers in material from lesions, is morphologically distinctive both in its

acid-fast staining character and the arrangement of the individual cells but has not, despite perennial reports to the contrary, been cultivated in the laboratory, either in lifeless mediums or in tissue culture, nor has direct inoculation of leprous material been unequivocally successful. There is an immune response in the disease, manifested as a hypersensitivity to soluble material (lepromin) extractable from leprous nodules. This last is feeble supportive evidence because the hypersensitivity crosses with that to tuberculin, but the leprosy bacillus is provisionally accepted as the etiological agent of the disease, largely because there appears to be no alternative agent.

The spirochete of syphilis also has not been cultivated in compliance with the second postulate, but its etiological relation to the disease is more firmly established because supportive evidence is relatively strong. This includes a specific immune response to the spirochetal substance appearing during the course of the disease and the reproduction of the disease by direct inoculation of infectious material containing, so far as is known, only the spirochete. In some instances, then, the requirement of the second postulate may be circumvented with a greater or lesser degree of probability of the presumed etiological relation.

In the case of the rickettsiae and viruses, isolation is necessarily growth in tissue culture or the embryonated hen's egg. It might be supposed that the purity of such cultures is questionable but, while this may be true to some extent, the occurrence of viruses in mixed culture in tissue culture is usually evident. Growth of these agents is directly demonstrable in the case of the rickettsiae, which may be seen in the light microscope, and indicated in the case of the viruses by appropriate, often characteristic, pathology of the tissue substrate, or some unique feature such as the development of hemagglutinin in the allantoic fluid of the embryonated hen's egg infected with viruses of the influenza group. In some instances, however, there may be no overt evidences of growth, and the agent may be carried by so-called "blind passage" in a series of tissue or egg cultures. After several such transplants cytopathogenic effects may become apparent, and in any case the material is tested for infectivity when the dilution is sufficient to rule out persisting infectivity. The somewhat devious nature of such procedures does not invalidate them.

The third postulate. The third postulate requires that the disease be reproduced in a susceptible animal by inoculation with the microorganism in pure culture. This is often difficult to accomplish for two reasons. First the disease must be reproduced in some recognizable form, *i.e.*, will need to bear sufficient relation to the naturally occurring disease. Second, a susceptible animal may be difficult to find. These are not matters of great importance in the study of diseases of, for example, domestic animals, for the natural host may be the experimental host also. In the case of infectious diseases of man, however, some experimental animal must be found. The etiological agents of human disease are often closely adapted parasites, so much so that they will either not infect, or will not produce disease in an experimental animal that is recognizable as related to the naturally occurring human disease.

While in a general way certain of the monkeys and higher apes, such as chimpanzees, are more susceptible to infection with human pathogens than are, for example, rodents, susceptibility does not necessarily parallel phylogenetic relationships. The search for a suitable experimental animal becomes in large part a matter of trial and error and educated guess. In the 1930's, for instance, the only reasonably available experimental animal in which poliomyelitis could be reproduced was the rhesus monkey. One eminent investigator systematically inoculated all kinds of experimental animals that came to hand, and finally found that one strain of this virus could infect the cotton rat. Similarly, influenza could not be reproduced in experimental animals for many years until the similarity of the lesions of the lung produced in ferrets

by distemper virus to those of human influenza were noted, and it was found that human influenza could be reproduced in the ferret by intranasal inoculation.

Other diseases of man, such as gonorrhea and typhoid fever, have not been reproduced in ordinary experimental animals, but some, such as tuberculosis and diphtheria in the guinea pig, are reproduced with facility. The mouse is exquisitely susceptible to infection with the pneumococcus on parenteral inoculation, and man appears to lie midway between the mouse and the more resistant dog with respect to susceptibility to infection with this bacterium. While there are many others, these examples suffice to illustrate the somewhat erratic occurrence of susceptibility to infection with the pathogenic microorganisms.

The importance of reproducing infectious disease in an experimental animal cannot be overestimated. Unless the disease can be studied under the controlled conditions of the laboratory it must remain inadequately understood. It is equally obvious that unless this third postulate is fulfilled it is not possible to fully establish the etiological relation of a given microorganism to a given disease.

The fourth postulate. According to the fourth postulate, the microorganism must be found in the infection produced in the susceptible animal, and shown to be the same as that inoculated. If the first three postulates are met, the fourth ordinarily follows without difficulty. It is nonetheless necessary to show that the animal was in fact infected with the microorganism and that the disease or death so produced is specific in that it is a consequence of that infection rather than some intercurrent infection of the experimental animal. Rarely there is some difficulty in this connection. Certain of the tumors of virus etiology, for example, are transmissible by cell-free filtrates, but after several passages by tissue implantation it may not be possible to find the virus. The reasons for this are relatively complex and, in part at least, are technical in nature.

The "fifth postulate." Following the discovery of the bacterial exotoxins, it was suggested that a fifth postulate, namely that the disease should be reproduced in susceptible animals by inoculation of the cell-free products of microbial growth, might be added. This is applicable in only a minority of cases and, while it lends support to the identification of the specific etiology of a disease, is not essential to establishing that etiology beyond reasonable doubt.

Supporting evidence. Less direct but supporting evidence for the etiology of a given disease assumes significance when the evidence provided by the four postulates is equivocal. This has been implied above in relation particularly to inability to reproduce human disease in characteristic form in experimental animals. The most important of such supportive evidence is immunological in nature. Taking the example of typhoid fever, and neglecting the accidental human infections with pure cultures of the typhoid bacillus which satisfy the requirements of the third postulate, immunological evidence strongly supports the etiological relation of the microorganism to the disease; the disease may be prevented by immunization with vaccines prepared from cultures of the microorganism, and the naturally infected individual who recovers from the disease develops an immunity directed specifically toward the microorganism which is demonstrable in a number of ways. Or the indirect supporting evidence may be epidemiological in nature, serving to pinpoint a particular microorganism with respect to a given disease even though the disease cannot be reproduced experimentally.

Multiple Etiology. In naturally occurring disease more than one kind of microorganism may be concerned, and three kinds of such diseases may be distinguished. Under some conditions secondary infection is not uncommon, the primary invader serving to reduce the resistance of the host so that a second kind of microorganism, of limited pathogenic powers and often a part of the normal microbial flora of the host, may

produce disease. Staphylococcal pneumonia, for instance, is usually not a primary infection but is found to occur most often as a secondary infection, commonly as a sequel to influenza. Similarly, Proteus is of relatively limited pathogenic capabilities, but is potentiated by staphylococci, apparently a substance formed by staphylococci, so that it becomes a virulent, lethal agent.[1] Other kinds of diseases are characteristically mixed infections in that more than one kind of microorganism is concerned from the outset; thus, perhaps half a dozen species of bacteria are the causative agents of gaseous gangrene, occurring in various combinations but rarely alone. In both secondary and mixed infections the causative microorganisms are independent and individually capable of producing a disease condition.

The third kind of infection is that in which the etiological agent is not single but multiple, and the disease is a consequence of a synergistic relation between the microorganisms such that the combination has pathogenic capabilities that its components do not. Such multiple etiology becomes complex and is possibly more common than now appears. Two examples will suffice to illustrate it.

One is the now classic example of dual etiology of disease, that of swine influenza described by Shope. Here the two microorganisms are a porcine variety of the influenza bacillus, *Hemophilus suis* (*H. influenzae* var. *suis*), and a variety of the influenza virus. The disease swine influenza is not caused by either the virus alone or the bacterium alone, but only by a combination of the two. The other example is that of diphtheria of man. For 70-odd years there was no reason to believe that the microbial etiology of this disease was other than clearly and fully established. Not only was the diphtheria bacillus isolated and characterized in 1883, but the following year the powerful exotoxin of the microorganism, capable of producing the disease symptoms and pathology, was described. Subsequently antitoxin therapy and highly effective immunization with toxoid were developed. About 1950, however, it was found that only lysogenic diphtheria bacilli are toxigenic, and therefore pathogenic (Chap. 7), and it became clear that the etiological agent of diphtheria is, in fact, not a single microorganism but a combination of the bacillus and the appropriate bacterial virus.

It is not yet clear how common the phenomenon of true multiple etiology may be. There are a number of indications that the etiology of many of the infectious diseases may not be as firmly established as has been thought. For example, there seems to be little doubt of the pathogenicity of the hemolytic streptococci, and yet inoculation of human volunteers with freshly isolated cultures has often given negative results. Similarly, meningococcus meningitis was an important problem in military personnel during World War I but failed to materialize to a significant degree during World War II in spite of high rates of carriage of the microorganisms. The occurrence of infectious disease is a highly complex phenomenon, involving more than the nature and properties of the causative microorganism, but it is possible that observed discrepancies may be due in part to an oversimplification of its microbial etiology.[95]

MICROBIAL VIRULENCE[103, 108]

The term "pathogenicity" has been used above in the generally accepted sense of ability to produce disease. It varies widely, not only from one kind of microorganism to another, but also between strains of a single kind of microorganism. A number of other terms denoting disease-producing ability are

used. The most common of which is virulence. Here the terms "pathogenicity" and "virulence" are used in the sense suggested by Miles,[1] *viz.*, pathogenicity is taken to mean potential ability to produce disease and is applied to groups or species of microorganisms, while virulence is used in the sense of degree of pathogenicity within the group or species. In this way, it is possible to speak of avirulent, virulent, highly virulent, etc., strains within groups or species of microorganisms that are said to be pathogenic.[98]

It is self-evident that ability to produce disease on the part of a microorganism is expressed only when the disease is produced. Conversely, the resistance of the host (considered in the following section) is determinable only as a reaction to appropriate exposure to the microorganisms. While, then, the two are fundamentally inseparable, for practical purposes they may be measured by holding one relatively constant and varying the other.

Measurement of Virulence. The measurement of virulence, then, consists of testing for ability to infect, with observable consequences, "normal" animals such as a standard strain of mice. The consequence observed is ordinarily an all-or-none phenomenon such as death. On challenge inoculation of experimental animals with graded doses of the microorganism, or substances such as bacterial toxins, it is apparent that virulence (or toxicity, etc.) is inversely related to the size of the effective dose, *i.e.*, the smaller the dose required to kill, for example, the greater the virulence. This is the basis of measurement of virulence (or toxicity) by determination of the minimum lethal dose, the smallest dose required to kill a standard experimental animal.

If each dose is given to groups of experimental animals, ranging in size from

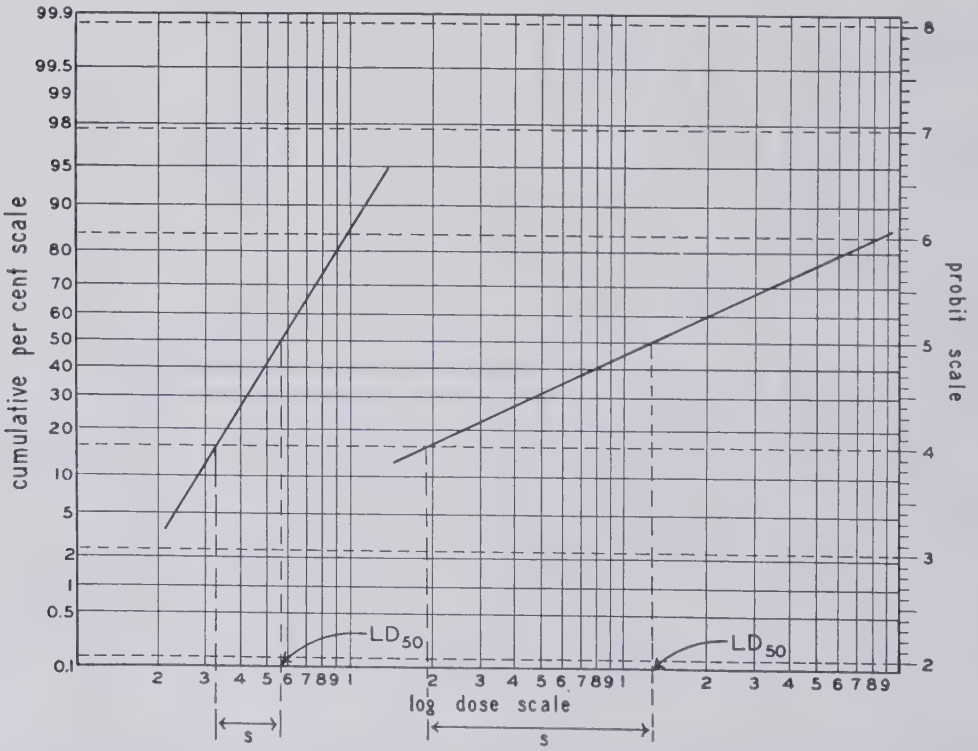


Figure 57. The graphical determination of the interpolated 50 per cent dose (ED_{50}, LD_{50}) and its standard error, s, on probability log paper. The vertical scale is the integral of the normal frequency distribution as per cent cumulative effect, and the horizontal scale is dose. The probit scale is on the right. Steep and less steep dose-response curves are shown.

a minimum of four to a practical maximum of ten to twenty, rather than individual animals only, two additional points are apparent. First, the proportion of animals reacting in each group varies from none or practically none in the group of animals receiving the smallest dose, to all or practically all of the animals in the group receiving the largest dose. The notation is usually fractional, the numerator the number of animals reacting and the denominator the number of animals inoculated with that dose, and recorded as 0/6, 1/6, 3/6, and 6/6, for example, for the several amounts of challenge inoculum.

Second, when the groups of animals are sufficiently large and the doses graded appropriately, it is apparent also that the proportion of animals affected per unit increment of dose rises slowly at first, then rapidly, and finally slowly again as the proportion becomes large. If the cumulative deaths or other effects are plotted against the logarithms of the doses, the points tend to fall on an S-shaped curve which represents the integral of the frequency distribution of natural resistance in the experimental animals used. When the cumulative effect is plotted on probability paper, on the scale representing the integral of the normal frequency distribution, against the logarithm of the dose, the points tend to fall on a straight line which may be fitted by inspection or by least squares.

The ED_{50}. The point on the dose scale at which this line intersects the 50 per cent point in the cumulative effect gives the interpolated dose which would produce the effect in half the animals inoculated with it. This is the 50 per cent effective or ED_{50} dose, or LD_{50} dose when death is the response measured. This value is, of course, subject to sampling error, and its precision is indicated by its standard error which is determined graphically also as indicated in the accompanying figure and is written as the $ED_{50} \pm$ the standard error. Such data allow the calculation of the significance of differences between titrations, as, for example, in protection tests (see below).[7, 8]

The standard error is the standard deviation or standard deviate of the normal frequency distribution and is the distance, in this instance on the dose scale, from center of the distribution to the point of inflection on either side and may, therefore, be either positive or negative to the central point. Since half the deviates are negative, the use of negative numbers may be avoided by adding the number 5, *i.e.*, at the central point the value is 5, at -1 it is 4, at $+1$ it is 6, and so on. This altered deviate is known as a probit. When the probit values for the cumulative percentage mortalities, or other effects, are plotted against dose on a logarithmic scale the relationship is linear because the probit scale is another way of representing the integral of the normal frequency distribution. The ED_{50} is the point on the dose scale at which the line fitted by inspection, or more precisely by the least squares method if appropriate, intersects 5 on the probit scale. The probit scale is superimposed on the probability scale in the accompanying figure.

The ED_{50}, or LD_{50}, measure of virulence is subject to qualification in that virulence is also indicated by the slope of the dose response curve. Slopes may vary widely from one pathogenic microorganism to another. When a highly virulent strain of pneumococcus is titrated in the mouse, for example, the dose response curve is steep and usually does not extend beyond a range of two logs on the dose scale. By contrast, when the virulence of Salmonella is titrated in the same animal, the dose response curve is much less steep, extending over as much as six logs on the dose scale. The significance of slope is not clear, but it is obvious that, to continue with the same example, the virulence of pneumococcus and that of Salmonella are not completely comparable as their respective LD_{50} values alone.

The dose-response curve, or the increasing proportion of deaths as the dose is increased, may be considered to be due to the independent activity of the self-reproducing particles, *i.e.*, to the increasing probability that a single effective microorganism will multiply to a

point at which death occurs. Quantitative studies substantiate this in highly susceptible animals, *i.e.*, when the LD_{50} is very small, but in partially resistant animals for which the LD_{50} is high, the total number of bacteria at death appears to be a consequence of growth of all the organisms inoculated rather than that of some effective portion.[59, 60, 62] The latter is consistent also with the generally observed inverse relation between size of inoculum and length of the incubation period.

Mucin. Many of the microorganisms pathogenic for man are so closely adapted to the human host that they are relatively avirulent for the usual experimental animals. It was first observed by Nungester that the virulence of many such microorganisms, *e.g.*, meningococcus, typhoid bacillus, dysentery bacilli, the cholera vibrio, can be markedly enhanced by inoculation of the bacteria suspended in gastric mucin. The role of mucin appears to be that of protecting the microorganisms against phagocytosis in the usual laboratory titration, but respiratory and alimentary mucus may function to protect the mucosae against infection in naturally occurring infections. Fractionation of this activity of mucins has been of some interest and a series of fractions have been obtained.[31] These include a heparin fraction which is also anticomplementary and associated with polysaccharide A, a viscosity fraction which is also a polysaccharide (polysaccharide C) fraction which acts synergistically with the heparin fraction, possibly affecting capillary permeability, and a particulate residue fraction which also acts synergistically with the viscosity fraction, but independently of the heparin fraction. Mucopolysaccharides of other origin, especially bacterial levans,[19, 93] also show some enhancement of virulence, presumably by lodgment in the interstices of capillary endothelium, thus modifying permeability and the inflammatory response.

The enhancement of bacterial virulence by mucin and similar substances, while of very considerable importance in making possible experimental infections otherwise obtainable only with great difficulty, derives its greatest significance from its implications concerning relevant facets of natural resistance.

Infections induced in this way are sufficiently artificial that, when they are used to assay the immune response by either the active or passive method, they give only results open to serious criticism. Obviously the protection demonstrable with such infections by antibody, chemotherapeutic drugs, etc., is a protection of the animal against the effects of mucin as well as against the microorganism.

Protection tests. The virulence titration is often used to assay the efficacy of some treatment such as active or passive immunization, or administration of drugs, by comparing, directly or indirectly, the LD_{50} of a given microorganism for normal animals and that for treated animals. From the analytical point of view, *i.e.*, the design of experiments, more reliable results may be obtained by using a standard challenge inoculum and varying the treatment. In measuring the protective capacity of immune serum by passive immunization of the animals to be inoculated, for example, it is preferable to administer varying amounts of antiserum to groups of animals, followed by challenge with some constant number of microorganisms. Similarly, in the active immunization test for the immunogenic potency of vaccines, groups of animals are given graded doses of vaccine followed by a constant challenge dose of bacteria after sufficient time to allow the immunity to develop. This preferred procedure is not always possible when the virulence of the challenge microorganism varies widely from one time to another. In such cases the protective material is given in constant dosage, and the LD_{50} dose is titrated in both treated and control animals, and protection measured by the ratio of the LD_{50} in treated animals to that in control animals.

The neutralization test, or serum neutralization test, is a variation on the protection test which is applied to the titration of antitoxin and antibody to

viruses. The toxin or infective virus preparation, previously titrated so that the dose is known, is mixed with varied amounts of antiserum, incubated for a time, and the mixture inoculated into susceptible animals. Survival of the animals is indicative of the presence of neutralizing antibody in the serum.

MICROBIAL TOXINS[40, 65]

The derangement of the normal physiological processes that is disease is, in the case of the infectious diseases, a consequence of the toxicity of proliferating microorganisms in the tissues. Toxic substances affecting tissues, cells, and possibly enzyme systems are formed by the microorganisms incidental to their metabolic activities which diffuse more or less freely from the microbial cells, and the cell substance of a wide variety of microorganisms is toxic also. In addition, toxic effects may be produced indirectly, as by the activation of tissue enzymes by bacterial kinases, or the formation of inflammatory and similar toxic substances by the affected tissues, and by the development of hypersensitivity (Chap. 14) to the microbial cell substance.

The toxicities of microbial origin may be separated into three groups, the potent exotoxins which are the classic bacterial toxins, the endotoxins contained within the intact cell, and a heterogeneous group of toxic substances, often enzymatic in nature, which diffuse more or less freely from the intact cell. Such a separation is artificial, in that lines of demarcation between the groups are often indistinct, but is useful for purposes of discussion.

Exotoxins

The bacterial exotoxins are the most potent poisons known, and it has been calculated that as little as 7 ounces of crystalline botulinum type A toxin would suffice to kill the entire human population of the world. These toxins have their counterparts in the less potent zootoxins such as the snake, spider, and scorpion venoms, and in the phytotoxins such as ricin and abrin. The formation of exotoxins by bacteria is relatively uncommon and is confined to the botulinus bacilli, the diphtheria bacillus, the tetanus bacillus, and one species, Shiga's bacillus, of the dysentery bacilli. The gaseous gangrene bacilli are sometimes regarded as producing exotoxin, but these activities are multiple in that the same strain of bacteria usually produces more than one toxicity, and these activities tend to fall into the group of enzymatically active toxicities.

Crystalline type A botulinum toxin is the most potent of the bacterial toxins; the LD_{50} for the mouse is only 4.5×10^{-9} mg. N. Type B botulinum toxin is somewhat less potent, 5 to 9×10^{-9} mg. N/LD_{50} for the mouse, and crystalline tetanus toxin 6.6×10^{-7} mg. N/LD_{50}. Highly purified but noncrystalline preparations of diphtheria toxin kill guinea pigs in amounts of 0.4 μg. per kilogram of body weight. The Shiga neurotoxin is of the same order of toxicity in purified form, but relatively small amounts are formed so that crude culture filtrates are not as toxic as those of the botulinus, tetanus, and diphtheria bacilli. While these amounts of toxin are extremely small, the toxins are neurotoxins and are not to be considered as diluted in the entire animal; the lethal dose of botulinum type A toxin for the mouse, for example, is about 20 million molecules, corresponding to about 8 molecules per nerve cell.

The potency of these toxins is, in general, paralleled by their efficiency as antigens. Antitoxic serums of high titer, 1 ml of which will neutralize thousands of guinea pig MLD's, may be obtained against diphtheria toxin. The neutralization of exotoxin by antitoxin proceeds according to the law of multiple proportions, *i.e.*, if x units of antitoxin neutralize y units of toxin, nx units of antitoxin will neutralize ny units of toxin.

The exotoxins appear to be proteins; they are denatured by heat, may be salted out of solution, etc. Type A toxin of *Clostridium botulinum* has

been prepared in crystalline form in two ways, using an initial acid precipitation followed by shaking with chloroform and salting out with ammonium sulfate, or a combination of sodium sulfate and acid precipitation. Both preparations crystallize in the form of needles and are pure protein, having the properties of globulin and a molecular weight of about 1×10^6. Type B botulinum toxin has been prepared as a pure homogeneous protein, though not crystallized. Tetanus toxin has been prepared in crystalline form by methanol precipitation in the cold and similarly appears to be pure protein. Diphtheria toxin has also been prepared in crystalline form following preliminary purification by adsorption of contaminants at an alkaline reaction by fractional precipitation with ammonium sulfate, phosphate, or citrate.[74, 75] These results definitely establish the protein nature of at least these bacterial exotoxins, but the crystalline materials are not necessarily homogeneous by immunological criteria, *e.g.*, antitoxin to crystalline diphtheria toxin contains a diphtheria bacillus agglutinin which can be removed by adsorption on the bacteria without reduction in antitoxic activity, and the crystalline toxin gives more than one line by the gel-diffusion precipitation method.

With the exception of botulinum toxin, the exotoxins are destroyed by proteolytic enzymes; botulinum toxin is, therefore, the only toxin which is effective when given by mouth.[50] There is no chemical evidence which explains, even in part, the toxicity of these substances; these proteins do not seem to differ in any essential respect from bland proteins such as egg albumin. The amino acid composition of crystalline type A botulinum toxin, for example, is in no way unusual. It has been suspected that toxicity might be a property of a prosthetic group attached to the protein molecule, but this appears not to be true; the balance of evidence indicates that the toxicity is a property of the structure of the toxin molecule, possibly of the arrangement of the constituent amino acid molecules in the protein.

The pharmacological action of the soluble toxins resembles that of the vegetable alkaloids and is sometimes quite definite and characteristic for each toxin. Diphtheria toxin, for example, produces degeneration of the heart muscles, kidneys, and liver and the hemorrhagic reaction in the adrenals which is a highly characteristic postmortem finding in the guinea pig.

The nature of the toxicity which results in these changes is not clear, but in this connection the implied nature of the toxin is of considerable interest. Largely through the work of Pappenheimer, considerations of the iron and porphyrin metabolism of diphtheria bacilli in relation to toxin production (Chap. 30) strongly suggest that diphtheria toxin may be the protein moiety of the cytochrome b of the diphtheria bacillus. This raises the interesting possibility that its toxicity may be related to the functioning of the respiratory enzymes of the host cells.

The activity of botulinum toxin has been studied in some detail. It has been found that acetylcholine produces contraction of botulinum-poisoned muscle but not of curare-poisoned muscle. This has been taken to indicate that acteylcholine is not produced in the end plates by the poisoned animal, and the action of the toxin is proximal to the point at which it is produced. Consistent with this, no acetylcholine is released during the neuromuscular paralysis produced by the toxin. The site of action of the toxin is on the nerve filaments, since acetylcholine is released following direct stimulation of the excised diaphragm of the guinea pig, but not following tetanization of the phrenic nerves. The neuromuscular paralysis occurring in the poisoned animal is a result of interference with conduction in the terminal twigs of motor nerves, near or at the points of final branching, but proximal to the site of acetylcholine release. The nature of the activity of the toxin that produces such interference with conduction is, however, completely unknown.

Tetanus toxin appears to act both centrally and peripherally. The nature of the

former action is not clear, but the latter effect seems to be twofold and includes a spastic action at the neuromuscular junction of voluntary muscles (local tetanus) and a paralytic action similar to that of botulinum toxin, *i.e.*, smooth muscles paralyzed by the action of the toxin on their cholinergic nerves still react to acetylcholine.

Following the injection of exotoxins, a period of incubation elapses before symptoms of intoxication appear. This incubation period may be very short or may extend to 36 or 48 hours or longer. The usual incubation period of tetanus toxin is 36 hours, but it may be reduced to 35 to 60 minutes by the injection of very large amounts, 500,000 MLD, of crystalline toxin.

The discovery that the toxic qualities of the soluble toxins are destroyed by treatment with formaldehyde which, at the same time, leaves the antigenic and antitoxin-combining properties of the toxin unimpaired, has been of the greatest practical importance in immunization procedures. Toxin so treated is called *toxoid*. It might be supposed that this observation would throw some light on the nature of the toxicity of these substances since formaldehyde is known to block amino groups. Analysis of the formaldehyde-protein derivatives indicates that the aldehyde combines with primary amino and primary amide groups but not with secondary amide or phenolic groups. Because of the effect of formaldehyde on toxicity and the results of detoxification with ketene and phenyl isocyanate, some workers have supposed that the free amino groups of the toxin molecule, such as the ϵ-amino group of lysine, are intimately associated with toxicity. There is evidence (Pillemer) which suggests that in the case of tetanus toxin, the toxoid is a dimer of toxin molecules condensed through their toxic groups.

Endotoxins[11, 32, 81, 104, 105, 111, 114]

The endotoxins contrast sharply with the exotoxins in that they apparently occur intracellularly in many gram-negative bacteria, especially the enteric bacilli, as lipid-polysaccharide-polypeptide complexes. They are heat-stable, *i.e.*, to boiling, in neutral solution but destroyed by mild acid hydrolysis, are not digested by proteolytic enzymes, are of relatively low toxicity with the mouse LD_{50} little less than 0.1 mg., are only partially neutralized by antiserum, and have substantially the same activity regardless of their origin. They appear to be structural components of the bacterial cell, represent the O antigen and occur, for the most part at least, in the cell wall of the bacteria.

The endotoxins may be extracted from the intact bacterial cell with trichloracetic acid or glycols or aqueous ethyl ether[78, 79] and are present in solutions of the cell substance prepared by mechanical disruption either before or after digestion with proteolytic enzymes. The complete complex is extracted in trichloracetic acid and in glycol, and in this form the polypeptide portion does not give the usual qualitative tests for protein. This kind of preparation is known as a Boivin, or Boivin-type, antigen since it was first prepared by the trichloracetic acid extraction method devised by Boivin. The bulk of the lipid fraction may be removed by treatment of the complex with hot formamide and is apparently unrelated either to toxicity or antigenicity. On further degradation the toxicity is often lost, and the free polysaccharide differs according to the kind of bacterium from which the endotoxin was prepared and is responsible for its antigenic character.

The nature of the toxicity is not clear, but Goebel and his collaborators showed that the endotoxin complex of the Flexner dysentery bacillus gave a nontoxic polysaccharide and a toxic acidic protein on acid hydrolysis, and on alkaline alcohol hydrolysis broke down to a toxic polysaccharide and a nontoxic protein. The toxic protein could be detoxified with alkaline alcohol, and the toxic polysaccharide by acid hydrolysis, but it was not possible to characterize the toxic element. Subsequently, a method of ex-

traction with 50 per cent phenol, and removal of the phenol by dialysis or extraction with an immiscible solvent, has been widely applied,[121, 122] which yields a lipopolysaccharide representing the endotoxicity. The small amount of contained lipid is distinct from the larger amount, 10 to 15 per cent, of the Boivin type of preparation and is not removed by extraction with appropriate solvents, and it is presumed to be an integral part of the toxicity. Such preparations may be taken to represent the toxic polysaccharides derived from Boivin antigen by various means.

On parenteral inoculation, the endotoxins produce a rise in body temperature, and are sometimes known as bacterial pyrogens, but their pharmacological activity is characteristic in the sense that they increase permeability. Capillary permeability may be increased with the production of local hemorrhage, and it is of interest that this occurs more readily in tumor tissue than in normal tissue so that if the dose is adjusted properly hemorrhage may be produced in tumor tissue only. The permeability effect is of interest also in that disease produced by many of the enteric bacilli is characterized by diarrhea. Following intracardial inoculation, grossly apparent edema and congestion with microscopic hemorrhages are produced in the small intestine within an hour or so.[9] A characteristic inflammatory response is given on intradermal inoculation, commonly of the rabbit, which may be used for titration of the endotoxic activity[53] by inoculation of endotoxin alone, or by potentiation with epinephrine.[113] The reaction to endotoxin is strikingly similar to the Shwartzman phenomenon (see below), and it is believed by some that this may account for some, or even a major portion, of their toxicity.

The resemblance of severe intoxication to shock is striking, with fall in arterial blood pressure and rise in venous pressure due to pooling of blood in the portal system, and coincident development of leucopenia and thrombodytopenia. It differs from anaphylactic shock in that clotting time does not increase.[119] Endotoxin shock may also occur in bacteremias due to gram-negative bacilli. As noted elsewhere (Chap. 7), these infections have become much more common with wide use of antibiotic therapy, and such shock becomes of considerable significance because it is often very difficult to differentiate from shock due to myocardial infarction.[120]

Contrary to the usual impression, the endotoxins are excellent antigens and stimulate the formation of agglutinating and precipitating antibody to high titer. The antibody, however, fails to neutralize the toxicity to more than a minor extent; the endotoxins thus resemble in this respect some artificially prepared pharmacologically active azo antigens whose activity is not neutralized by antibody, though they combine with it.

Viral and Rickettsial Toxins.[16] Toxic substances are associated with a number of viruses and rickettsiae which are endotoxins in the sense that they have not been dissociated from the infectious agent. While the pathological changes produced by these activities include effects on permeability, they are set apart from the bacterial endotoxins in that they are heat-labile and are neutralized specifically and more effectively, possibly in multiple proportions, by antiserum. Nothing is known as yet of their chemical nature.

Viruses of the psittacosis-lymphogranuloma group contain a labile toxicity demonstrable by intravenous inoculation of mice, but large doses, equivalent to 40 million infective doses, are required to kill. Mice dying early, *i.e.*, within 12 hours, show lung congestion with scattered hemorrhages, edema fluid in the alveoli, fibrin thrombi in the glomerular capillaries in the kidneys, and foci of damage or necrosis in the liver. In animals dying later, liver damage is the most pronounced feature with numerous necrotic foci accompanied by hemorrhage.

Influenza virus, in doses of 1500 to 2000 hemagglutinating units, produces similar changes in the mouse on intravenous inoculation, the more toxic strains producing effects on the intestine

such as edema and vascular engorgement. On intracerebral inoculation in the mouse, neurological signs are produced with tremor, clonic convulsions changing to tonic convulsions, and meningo-encephalitic changes in the brain. Intravenous inoculation of mice with Newcastle disease virus produces changes similar to those found with influenza virus, *e.g.*, lung consolidation and hemorrhagic enteritis.

Of the rickettsiae, those causing the typhus fevers and scrub typhus, and possibly also spotted fever, form similar toxins. In the mouse and rat, engorgement of visceral blood vessels and hemorrhagic enteritis with accompanying edema are common features of the acute intoxication. More detailed studies have shown that, shortly after inoculation, vasoconstriction with leakage of plasma and altered capillary permeability occur in the mouse.[117] In addition, the toxin of the rickettsiae, of endemic typhus at least, produces extensive intravascular hemolysis in the rabbit.[67]

It is not clear to what extent viral and rickettsial toxins may contribute to the disease process. For example, in one cycle of influenza virus development, forty to sixty infective particles are liberated from the infected allantoic cell. In volume these represent only a minute portion of the total volume of the cell, perhaps 1.5×10^{-5}, and yet it is sufficient to cause death. The implication would seem to be that replication involves some vital portion of the cell, for the postulated toxicity of the virus to produce death is improbably high. On the other hand, accumulated toxicity may well be a contributing factor in, for example, altering capillary permeability.

Other Toxic Substances

In addition to exotoxins and endotoxins, a variety of other substances are formed by microorganisms which may contribute to the disease process, either directly or by facilitating the establishment of a focus of infection. Some of these are cytotoxic, affecting the red and white blood cells, others interfere, directly or indirectly, with the clotting mechanism, and still others are enzymes catalyzing the decomposition of structural elements of the tissues such as the hyaluronic acid ground substance and collagen. The more important of these activities may be considered briefly.

Hemolysins. A variety of bacteria produce hemolysins, substances which bring about the dissolution of the red blood cells of higher animals. The bacterial hemolysins, which are to be distinguished from the immune hemolysins formed by an animal in response to the injection of red cells of another species, are of two types, the so-called filterable hemolysins which are extracellular and may be separated from the bacterial cells by filtration and the hemolysins which are demonstrated by the cultivation of bacteria on semisolid mediums containing whole blood.

Filterable hemolysins. The filterable hemolysins are sometimes named after the bacteria which form them; a *streptolysin,* for example, is a hemolysin produced by streptococci, and a *staphylolysin* a hemolysin of staphylococci. Hemolytic activity is demonstrated by the addition of filtrate or whole culture to a suspension of washed erythrocytes in physiological salt solution; after a period of incubation the red cells are laked, and hemoglobin appears free in solution. The relation of these hemolysins to other naturally occurring hemolysins such as saponins, the hemolysins present in snake venoms, and the like, is uncertain.[73] The bacterial hemolysins appear to be proteins in nature, are inactivated by heating (55° C. for 30 minutes), and are antigenic, *i.e.*, when injected into animals they stimulate the formation of antihemolysins.

That hemolytic activity is a property of a group of substances of bacterial origin rather than of a single substance formed by a number of bacterial species is indicated not only by differences in immunological specificity but also by the varied properties of the activity. Many hemolysins, for example, are oxygen-stable, but some, produced by the pneu-

mococcus, certain strains of streptococci, and some of the sporulating anaerobes, are oxygen-sensitive, *i.e.*, they are active in the reduced form but inactive in the oxidized form, the oxidation-reduction being reversible at low temperatures. Hemolysins further differ from one another in their heat and acid resistance and in the incubation time which precedes visible laking of the red cells. Differences in their activity on the erythrocytes of various species of higher animals may be marked; a given hemolysin, for instance, may lyse sheep cells but not rabbit cells. In general, the cells of the animal species from which a bacterium is isolated are more sensitive to its hemolysins than are the cells of other animal species.

A single bacterial strain may form more than one hemolysin. Certain strains of staphylococci form at least two hemolysins, one acting on both rabbit and sheep cells and bringing about a rapid lysis at 37° C., and the other acting only on sheep cells which are lysed after holding at room temperature overnight—a phenomenon which has been termed "hot-cold" lysis. The former is termed α lysin and the latter β lysin. The action of both can be shown in culture on sheep blood agar, the α lysin producing a zone of complete clearing on 24 hours' incubation and the β lysin producing a zone of darkening which becomes lighter and clear on subsequent refrigeration of the plate. Staphylococci produce still other hemolysins in addition to these (Chap. 15).

The occurrence of multiple hemolysins and hot-cold lysins is by no means limited to the staphylococci. Many streptococci, for example, produce two hemolysins, an oxygen-labile heat-stable hemolysin called streptolysin O and an acid-sensitive heat-labile streptolysin S. The occurrence of multiple hemolysins is especially marked among the sporulating obligate anaerobes making up the gas gangrene group. For instance, the most frequently occurring of these, *Clostridium welchii,* produces three toxins, the α, θ, and κ toxins, of which the first two are hemolysins, and the α toxin is a hot-cold lysin.

The production of hemolysins is sometimes favored by inclusion of serum in the broth in which the microorganisms are cultured, and it is of some interest that the activity of the hemolysin formed in such mediums is most marked on the red cells of the animal species from which the serum was derived. The hemolytic activity of a culture may be transitory, probably because of inactivation of formed hemolysin. Some hemolysins lose their activity when incubated at 37° C. for 2 hours or more.

The precise manner in which the structure of the erythrocyte is altered so that contained hemoglobin is released is not clear, but it is an all-or-none phenomenon in that a single cell is not partially lysed. Like certain other hemolytic agents, the bacterial hemolysins apparently alter the surface of the cells, and swelling occurs prior to lysis.

Blood-plate hemolysis. The colonies

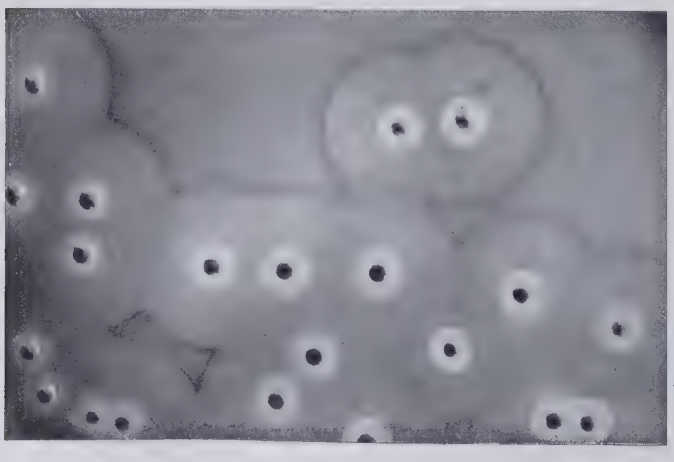


Figure 58. "Hot-cold" hemolysis by *Staphylococcus aureus* on sheep blood agar, produced by holding the plate alternately at incubator and refrigerator temperature. The small clear zones are produced in the incubator by the α lysin and the large zones in the refrigerator by the β lysin.

of some bacterial species on blood agar produce visible changes in the medium immediately surrounding the colony which are termed "hemolysis." Two general types of change are observed, one designated as α or green hemolysis, in which the bacterial colony is surrounded by a zone of greenish discoloration, and the other, β hemolysis, in which the zone around the colony is clear and uncolored, in contrast with the red opacity of the medium. Microscopic examination of the green zone shows the presence of many discolored corpuscles, but in β-hemolytic zones corpuscles cannot be found.

The relation between blood-plate hemolysis and the filterable hemolysins is usually regarded as uncertain, for microorganisms hemolytic on blood plates may not appear to produce filterable hemolysin; some workers maintain that, since filterable hemolysins are difficult to demonstrate, failure to find them is not significant. It would appear that the processes involved are different; in the case of the filterable hemolysins the permeability of the red cell is altered and the hemoglobin escapes into the surrounding fluid, while in blood-plate hemolysis the pigment is broken down to green or colorless compounds. In the case of the cholera vibrio the two processes have been sharply differentiated, since the identification of this organism is dependent in part on the hemolysis of goat erythrocytes in suspension (Greig test); though the true cholera vibrio is negative to this test, some strains show hemolysis, or hemodigestion, on blood agar plates (Chap. 23). On the other hand, there appears to be a close association between β hemolysis and streptolysin O.

The mechanism of β hemolysis is not well known, but in the case of staphylococcal hemolysins, the hemoglobin is not destroyed; according to Christie and Graydon the clearing around the colony is due to migration of the liberated hemoglobin. Green hemolysis was formerly thought to be due to the formation of methemoglobin and associated with the formation of hydrogen peroxide, but it has been found that the green substance is an iron-containing derivative of hemoglobin possibly formed by reduction. As in the case of the filterable hemolysins, hemolysis on blood agar is frequently species-specific; a higher proportion of bacterial strains will show hemolysis on blood from one animal species and not on that of another.

The relation of ability to form hemolysin and virulence is obscure. Although among some of the parasitic bacteria such as streptococci and staphylococci virulence is associated with hemolytic activity, a number of saprophytic bacteria also produce hemolysins. On the other hand, it has been reported that in staphylococcus infections of the udder, the hemolytic strains are much more irritating than the nonhemolytic organisms. It might be expected that infection with actively hemolytic bacteria would be accompanied by anemia and hemoglobinuria, but this is not the case as a rule; infections with *Cl. welchii*, however, are frequently characterized by gross blood destruction and consequent severe anemia and jaundice which is due to the continuous release of the hemolytic α toxin. The massive intravascular hemolysis and associated hemoglobinuria, together with hyperkalemia, produced by the toxin of scrub typhus rickettsiae referred to above, may be a significant part of the pathological picture of this disease. In many instances, of course, a bacterial hemolysin may be toxic in other ways, *i.e.*, bacterial toxins are sometimes hemolytic.

Leucocidins. A number of bacteria, notably staphylococci, streptococci, and pneumococci, form leucocidins, substances which kill and, in some cases, lyse, polymorphonuclear leucocytes. The death of the leucocytes results in the inability of these cells to reduce methylene blue, a phenomenon made use of to demonstrate the activity of these substances.[57] A direct microscopic method using phase contrast microscopy has shed some light on the effect of these substances on the white blood cell. The leucocidins so demonstrable are the so-called Panton-Valentine (PV) leucocidins, for it is believed by some that leu-

cocidal activity shown in different ways is not necessarily a measure of the activity of the same leucocidin. Human and rabbit leucocytes in contact with leucocidin become spherical; the nuclei fragment and the cells may, with some leucocidins, eventually burst or, with others, remain intact.

The amount of leucocidin produced by one bacterial species may vary widely from one strain to another, and a single strain may produce more than one leucocidin. The detection of more than one leucocidin is dependent upon differences in thermostability and in the kind of leucocytes attacked; one staphylococcal leucocidin, for example, affects both rabbit and human leucocytes, while another is active only on rabbit cells. In general, the leucocidins closely resemble the filterable hemolysins in that they are antigenic, variable in their heat resistance, and so on, and, in some cases, may be identical with hemolysins. The α hemolysin of staphylococcus, for example, is a leucocidin. They may not be strain-specific; some of the staphylococcal leucocidins, for example, are immunologically similar if not identical regardless of the bacterial strain from which they are derived.

The part played by the leucocidins in the virulence of a bacterium is obscure. Theoretically, it should be of some advantage to an invading microorganism to be able to destroy these phagocytic cells and thereby break down, to some degree, the defenses of the body. The polymorphonuclear leucocytes are not, however, the most important cells in the phagocytic destruction of bacteria, and the effect of these substances on the other phagocytic cells, the mononuclears, the histiocytes, and others, is not well known. As in the case of the hemolysins, the leucocidins of bacterial origin must be differentiated from the immune leucocidins, those produced by the animal body in response to the injection of leucocytes from another species.

Coagulase. The formation of blood clots is accelerated by coagulase, a substance of bacterial origin. The production of coagulase is apparently confined to the staphylococci for the most part and may be causally related to thrombus formation that is common in infections with these organisms. Occasional strains of other bacteria such as *Pseudomonas pyocyaneus, Serratia marcescens, Escherichia coli,* and *Bacillus subtilis* have, however, been observed to accelerate the clotting of blood. Coagulase activity is demonstrated by the addition of bacteria to citrated or oxalated blood plasma (human or rabbit) which gels within three hours. It is also demonstrable by mixing the bacteria with plasma on a slide and examining for the clumping that occurs with fibrin formation—the plasma-clumping test.

It has been found that the formation of a clot occurs in two stages, *viz.,* a reaction between the substance produced by the bacteria, procoagulase, with a cofactor or activator present in plasma to form the clotting agent, coagulase, and clotting of the plasma under the influence of coagulase. While, then, strictly speaking, the bacterial factor is procoagulase, it is commonly referred to as coagulase. It is relatively heat-stable and may be obtained cell-free with some difficulty. Its antigenicity has been open to some question, but apparently has been established. The plasma factor is closely similar to, but not identical with, prothrombin from which it may be separated by Seitz filtration (prothrombin is retained) and is associated with the globulin fraction. There is some evidence that coagulase occurs in two forms, one bound to the cell and the other free;[23, 24] the former is presumably involved in the plasma-clumping test.

The distribution of the plasma factor among animal species accounts for the differences in the clotting behavior of plasma; most coagulases clot human, rabbit, and horse plasma, but not rat, chicken, or guinea pig plasma, though the last may clot slowly at 25° C. but not at 37° C. It is generally agreed that the formation of coagulase by staphylococci is closely associated with virulence, and reference is commonly made to "coagulase-positive staphylococci," implying

virulent staphylococci. There is an association between the susceptibility of animals to staphylococcal infection and formation of the plasma cofactor. Thus rats, mice, and guinea pigs, are usually relatively resistant to experimental infection with coagulase-positive staphylococci, and the virulence of coagulase-positive staphylococci for such animals can be enhanced by inoculating with bacteria suspended in coagulable plasma. Apparently bacteria coated with a film of fibrin are phagocytosed with difficulty, and, in addition, the clumping of both bacteria and phagocytic cells by fibrin tends to inhibit mechanically the process of phagocytosis. Further, it has been observed that coagulase antagonizes, to a greater or lesser extent, the antibacterial activity of normal serum, and coagulase-positive bacteria are able to grow in normal serum while coagulase-negative are not.[25]

The Bacterial Kinases. A number of kinds of bacteria, notably the streptococci, are able to dissolve fibrin clots or inhibit the clotting of plasma. This activity was described by Tillett and Garner in 1933 as fibrinolysin. It may be titrated as the highest dilution that will lyse a fibrin clot in a given time, or as the time required to lyse a fibrin clot, or, finally, by the amount of protein made soluble in trichloracetic acid. The last is the least sensitive because dissolution of the clot occurs earlier than proteolysis to trichloracetic acid–soluble substances or free amino nitrogen.

The reaction does not occur with a clot formed by the interaction of purified human thrombin and fibrinogen. It has been shown that the factor produced by streptococci is not in itself the lytic substance, but a kinase that activates a precursor (plasminogen) of a plasma protease (plasmin) occurring in the euglobulin, and that the plasmin is actually the lytic agent. The bacterial activity, then, is not a fibrinolysin as it was originally and still is called, and is more properly referred to as a kinase, streptokinase in the case of the streptococcal activity.

Streptokinase. There are certain anomalies in the bacterial activity. For example, streptococci of human origin will lyse human plasma clots, but the plasma of most animals is not susceptible. Animal fibrin is, however, susceptible if clotted with human thrombin, and human fibrin is susceptible if clotted with animal thrombin. Moreover, rabbit clots become susceptible if mixed with plasminogen of human origin. Such observations are in large part explained by the occurrence of both free plasmin and a plasmin inhibitor, antiplasmin, in plasma, the latter in the albumin fraction. Thus the insusceptibility of rabbit plasma is due to the presence of relatively large amounts of antiplasmin rather than a deficiency in plasminogen as once thought. Similarly, on dilution of plasma, the plasmin-antiplasmin complex dissociates, and the solution becomes fibrinolytic. It may be noted parenthetically that inactivation of antiplasmin by chloroform is the basis of a commercial purification of antiserum; the antiserum is shaken with chloroform and incubated to allow the preferential digestion of serum proteins other than antibody globulin to occur.

Fibrinolytic, or lysokinase, activity occurs among bacteria other than the streptococci. That of staphylococci, staphylokinase, is somewhat different from the streptokinases in species specificity and other respects; there is some evidence that staphylococci that produce β lysin (hemolysin) do not produce staphylokinase. A variety of other bacteria, including enteric bacilli and some of the sporulating obligate anaerobes, digest fibrin clots, but for the most part not a great deal is known about these activities and possibly in some instances the digestion is due to proteolytic enzymes rather than kinases.

The streptokinases are antigenic and those from different strains appear to be very similar immunologically. Antiserums specifically inhibit the activity, and the occurrence of antikinase in the serum has been useful as an indicator of past infection with kinase-positive streptococci.

A number of bacteria exhibit a tend-

ency to inhibit the clotting of plasma, a property that may be demonstrated by delaying the addition of calcium to the oxalated plasma; when calcium is added after a period of preliminary incubation, the clot fails to form. This property has been most often demonstrated with cultures in dextrose broth, and, in some cases at least, the failure of the plasma to clot is a result of the presence of lactic and other acids formed in the fermentation.

Streptokinase appears to be closely associated with virulence and particularly with the ability to invade the body tissues. Since one of the first reactions of the body to tissue destruction is the formation of blood clots which tend to wall off and isolate the infected region, it is not surprising that a bacterium capable of lysing these clots should show marked tendencies to extensive tissue invasion. The streptococci are among the most invasive of the pathogenic forms, and this phase of their virulence may be attributed in part to the formation of fibrinolysin.

Hyaluronidase (Spreading Factor, Invasin).[22, 58] The permeability of the tissues is remarkably increased by a factor, often called the *Duran-Reynals factor* after its discoverer, present in certain mammalian tissues, notably the testes. Bacteria, vaccinia virus, and substances such as toxins, India ink, and the like, diffuse rapidly from the site of inoculation when injected in conjunction with extracts containing this factor. Strains of staphylococci and streptococci that have no pronounced invasive powers may, in this way, be rendered highly invasive, and hyaluronidase is apparently essential for producing inhalation infection.[18, 44] This factor is also present in a number of bacteria notable for their invasive properties, such as certain strains of staphylococci and streptococci, pneumococci, and certain of the obligate anaerobes such as the bacillus of gas gangrene. Among some of these organisms there is some degree of association between content of the Duran-Reynals factor and virulence; the noninvasive strains of staphylococci noted above contain little or none of this factor, but become invasive when it is supplied, while the invasive strains produce the factor themselves.

This substance is an enzyme, hyaluronidase, whose substrate is hyaluronic acid, a mucopolysaccharide consisting of acetylglucosamine and glucuronic acid, which acts as a cement substance of the tissues and is found in synovial fluid and elsewhere in the body.

Decomposition of hyaluronic acid occurs in three stages, *viz.*, in the first it is no longer precipitable with acetic acid, the second stage is that of depolymerization and coincident decrease in viscosity, and the third is characterized by the liberation of reducing sugars. Each of these has been used for assay of hyaluronidase activity, and most *in vitro* methods are based on viscosity changes. The action of the enzyme in decreasing the viscosity of hyaluronic acid by hydrolysis is responsible for facilitating the penetration of the tissues by bacteria that produce it. The effect on the tissues is temporary in that, following inoculation of preparations of the enzyme, the dermal barrier is restored partially in 24 hours and completely in 48 hours.

It is of some interest that hyaluronic acid is also found in the capsular substance of some strains of streptococci. Such strains do not produce hyaluronidase and in its presence are denuded of capsules and more readily phagocytosed (see below). The ability of such strains to invade the tissues does not, of course, depend upon their elaboration of hyaluronidase. The activity appears to be antigenic, in that it is neutralized by antiserums, but immunologically distinct from different sources; it has been suggested that the enzyme is combined with different proteins in different organisms.

Hyaluronidase is antagonized by an enzyme present in normal blood plasma which has been called anti-invasin I. An enzyme in bacteria, pro-invasin I, destroys anti-invasin I, and another enzyme in plasma, anti-invasin II, destroys pro-invasin I. It has been suggested that a

balance between all these in the host-bacterial system determines whether or not invasion of the tissues will occur.

Streptodornase. Streptodornase is a deoxyribonuclease produced by many hemolytic streptococci, together with streptokinase, which depolymerizes viscous DNA, and its activity may be titrated by viscosity methods. It appears to be unrelated to virulence. Both streptodornase and streptokinase are available for the enzymatic debridement of wounds. The functioning of the latter depends upon the presence of sufficient plasminogen. Plasminogen is not available generally for this purpose, but may be prepared from human plasma.[15]

Capsules

The association between the presence of a capsule on a pathogenic bacterium and its virulence has been discussed elsewhere (Chaps. 3 and 7). The capsular material, generally polysaccharide in nature although nitrogen and amino acids may be present, is not in itself toxic and cannot be regarded as analogous to toxins, hemolysins, and similar substances. Rather the capsule appears to function as a defensive mechanism of the bacterium against the phagocytic activity of the leucocytes. Encapsulated bacteria may be ingested by a white blood cell but, instead of being killed and digested, remain within the phagocyte for a time and then may be extruded in a viable condition. The ability of an encapsulated bacterium to resist phagocytic destruction may, in fact, result in a wider distribution of the microorganism than it might otherwise attain, through transport within the phagocytic cell. It is of some interest in this connection that nonencapsulated, avirulent pneumococci are highly virulent for rabbits deprived of their leucocytes. It is perhaps suggestive that the polypeptide material making up the capsule of the anthrax bacillus contains D(—) glutamic acid. Since proteolytic enzymes attack only polypeptides built up from amino acids of the L-series, possibly an encapsulated anthrax bacillus would be highly resistant to the digestive enzymes of a phagocytic cell.

Whether the association of virulence and capsule formation may be entirely accounted for on the basis of bacterial defense against phagocytic destruction is not entirely certain, but such defense undoubtedly plays an important part. The presence of antibodies to the capsular substance breaks down this bacterial resistance, and immunization to the capsular material of the pneumococcus, for example, produces just as high a degree of immunity to pneumococcus infection as immunization to the entire bacterium.

Miscellaneous Factors

In addition to these more or less well defined and better known factors, a number of bacteria have been reported to produce substances which may be associated with virulence. Such, for example, are the necrotizing factor, or necrotoxin, produced by some staphylococci, which kills tissue cells; a hypothermic factor produced by Shiga dysentery bacilli, which lowers body temperature; an edema-producing substance formed by pneumococci; substances associated with the endotoxins of some of the enteric bacteria which affect the blood sugar levels of animals, and so on. Unfortunately, the discontinuous character of present information does not support any satisfactory generalization regarding bacterial virulence, but it seems clear that a pathogenic bacterium may have at its disposal a series of mechanisms, in combination peculiar to itself, which make possible a successful invasion of the tissues of the host.

Toxicity of Host Origin

It is not uncommon to observe signs of toxemia in infectious disease when the causative microorganism cannot be shown to form toxic substances *in vitro*. The classic example of this is the marked

toxemia of acute pneumococcal infection, while the pneumococcus cell substance and metabolic products formed in culture are comparatively bland. Observations of this kind suggest either that toxicity is of host origin in that it is produced by the infected host tissue or that the microorganism produces toxic substances in the environment of the infected tissue but not in culture *in vitro*.

There are two obvious alternatives in the former. It may be postulated that, in the interaction of the metabolic systems of the proliferating microorganism and the host cells, those of the latter are perverted with the accumulation of substances toxic to the host. While there is no definitive evidence of such interaction, the plausibility of the hypothesis is enhanced by the relatively rapid disappearance of signs of toxemia on institution of chemotherapy. The chemotherapeutic drugs are not antitoxic, though some small effects on microbial toxins have been described, but they inhibit proliferation of the microorganism, suggesting that the active metabolism of the growing microorganism may be associated with toxicity.

The other alternative is a more or less specific interference by a bacterial product with a functional system of the host. Two examples of this have been described above. One is the activation of plasminogen by the antagonism of the bacterial kinases, such as streptokinase, for its normal inhibitor, antiplasmin. The other is the staphylococcal product, inaccurately termed coagulase, which activates a plasma cofactor separable from prothrombin. In some instances it may be shown that toxic substances are produced by the host tissue. The thromboplastic toxin, or necrosin, that may be isolated from skin lesions of streptococcal etiology may also be found in normal skin subjected to traumatic injury.[17, 89]

Other phenomena of this kind are less well understood. There is, for example, good evidence that the febrile response to bacterial substance often is not due to pyrogenic substances present in the microorganisms but is a consequence of an interaction between the bacterial substance and plasma to form, or liberate, pyrogenic substances of endogenous origin.[36] Still another such phenomenon is the greatly increased sensitivity to histamine, on the order of a hundredfold, that is developed following inoculation with pertussis bacilli, that is perhaps attributable to an effect on the rate of histamine release from the tissues. This kind of "hypersensitivity" is possibly related to the pathogenesis of whooping cough in which the "whoop" persists after the bacteria can no longer be found in the infected individual. In this connection it is suggestive that the sensitivity to histamine so produced is more marked in female than in male mice, and in whooping cough the general rule that mortality from infectious disease is significantly greater in the male is reversed.

The Shwartzman Phenomenon.[94, 115] The reaction known as the Shwartzman phenomenon may be regarded as a special case of a host reaction resulting in increased sensitivity to microbial substances. It occurs most readily in the rabbit and is produced by two inoculations, a "preparatory" inoculation and a "provocative" inoculation, a few (8 to 30) hours apart, and the reaction so produced may be local or general. In the former, the preparatory inoculation is given intradermally, and the provocative inoculation intravenously; a short time after the second inoculation gross hemorrhage and necrosis develop at the site of the preparatory inoculation. The general reaction is induced by giving both inoculations intravenously and is characterized by bilateral cortical necrosis of the kidneys. While the time element and the lack of necessity for a common antigenicity in the preparatory and provocative substances precludes an immunological basis for the reaction, it resembles overtly the Arthus phenomenon (Chap. 14) and is intensified when the animal has received immunizing inoculations of the preparatory material.

Not all bacteria contain preparatory substances, but the endotoxins of the enteric bacilli are almost uniformly effec-

tive. It has, in fact, been suggested, especially by Stetson, that the toxicity of many bacterial endotoxins may be a manifestation of the Shwartzman reaction, possibly enhanced by an immune response in some instances, in which the preparatory dose is absorbed from the bowel as endotoxin of coliform and related components of the normal intestinal flora. There is a wider range of provocative than of preparatory substances, and substances such as starch or agar, serum, or bacteria which have no preparatory effect may be provocative.

The participation of the host in this reaction is indicated in a number of ways. Following the preparatory intradermal inoculation, there is a local cellular reaction around the venules in the area, an increased glycolysis associated with the influx of heterophils, and a local accumulation of lactic acid. This response, and subsequent reaction to the provocative inoculation, is prevented by treatment which induces leucopenia, such as x-irradiation or the administration of nitrogen mustards. The significance of the cellular response is also indicated by the enhancement of the reaction when the macrophage system is blockaded with Thorotrast and an increased sensitivity to endotoxin preparatory inoculation when the animal is treated with cortisone. In total, the evidence suggests the preparatory inoculation interferes with a significant detoxifying mechanism.

Local infection with a variety of bacteria, such as tubercle, anthrax, and influenza bacilli and streptococci, are preparatory even though the microorganism itself may not be preparatory. Thus streptococci are not preparatory though a local streptococcal infection may be, and preparatory activity may be demonstrated in extracts of the infected tissue. The extent to which the Shwartzman type of reaction contributes to the pathogenesis of infectious diseases is, however, not at all clear.

Microbial Toxins Formed in Tissues. The second possibility alluded to above is that of the production of toxin by the microorganism only in the environment provided by the infected tissue. This is suggested by, for example, the increased virulence that may be induced by animal passage, the inability to differentiate *in vitro* between virulent and avirulent strains of certain microorganisms such as the plague bacillus, and by the contrast between anthrax bacilli *in vitro* and *in vivo*.

The last is one of the most thoroughly studied instances of this kind. It is considered in more detail elsewhere (Chap. 28) but may be noted briefly here for illustrative purposes. There is little or no evidence of toxicity of the usual *in vitro* culture of the anthrax bacillus, nor can effective killed vaccines be prepared from such cultures even though the bacilli may be shown, by animal inoculation, to be highly virulent and to produce an immunizing infection. While a number of factors are involved, it suffices for present purposes to note that an effective immunizing antigen may be extracted from infected tissue, and elucidation of the nutritive requirements for its production have allowed the development of culture mediums in which it is formed *in vitro*. This immunizing substance is not of sufficient toxicity to account for the death of infected animals, but such toxicity is demonstrable in the plasma of infected animals. This toxicity is separable into two fractions, one of which is sedimented by high speed centrifugation while the other is not. Separately the two are only moderately toxic, but when combined their toxicity is synergistic. The sedimentable fraction resembles the immunizing antigen, differing in that it is toxic when combined with the nonsedimentable fraction, and the immunizing antigen may be a "toxoided" form of the toxic fraction. It is not yet known in what way the tissue environment makes possible the formation of these toxic substances, but it is clear that the toxigenicity of these bacteria, and no doubt others also, in the infected animal is not adequately reflected in the toxic activities of *in vitro* cultures.

RESISTANCE [26, 28, 29, 64, 92, 99, 109]

It is already apparent from the foregoing considerations of microbial virulence that, although the ability of a microorganism to produce disease is conditioned by a series of mechanisms originating with the microorganism, pathogenicity must be evaluated in terms of the resistance of the host. As a rule, a pathogenic microorganism is limited to a small number of hosts; microorganisms pathogenic for animals are not ordinarily pathogenic for plants; very few of the microorganisms that can infect mammals are also pathogenic for cold-blooded animals; some are even restricted to the tissues of a single species. Resistance, like virulence, is made up of many factors, some of which are known either in more or less specific form or in the terms of generalities that serve as a cloak for ignorance; others are, in all probability, as yet unsuspected. Resistance to infection is, in a sense, somewhat more complex than virulence for, as will appear, not only are there specific barriers to infection variable with respect to species and even from one tissue to another in the body of a single animal, but the efficiency of these barriers is also a manifestation of general physiological well-being, and hence they are subject to extrinsic or environmental stress.

The factors operable in the host are of two general kinds, the constitutive group, which includes those which occur in the normal animal, and the adaptive group, which are the responses to the presence of the pathogenic microorganism. The latter are predominantly, though not entirely, those associated with the immune response, and are considered elsewhere (Chaps. 13 and 14), while the former are discussed here.

Species, Racial, and Inherited Resistance.[34, 86, 87, 110] Species of higher organisms differ greatly from one another in their resistance to any given disease, a fact that has been suggested earlier in connection with the experimental reproduction of disease. In many cases resistance to infection is relative, for disease may sometimes be produced by the administration of massive doses of bacteria to a resistant animal, but in others it appears to be absolute. Man is, for example, apparently completely immune to cattle plague, and many of the lower animals are equally resistant to some diseases of man. In general the factors underlying differences in species resistance are unknown, but in a few cases body temperature or differences in anatomical structure have been found to account for the observed variation. Pasteur's classic experiment in which he rendered the naturally resistant hen susceptible to anthrax by chilling it in cold water, and the converse of this experiment, the production of anthrax in the resistant frog by raising its body temperature from 25° to 35° C., may be explained in terms of unfavorable body temperatures. Similarly, cold-blooded animals are not susceptible to human tuberculosis, and warm-blooded animals are not infected by the tubercle bacilli of cold-blooded animals.[6, 83] The insusceptibility of experimental animals such as guinea pigs and rabbits to the enterotoxin produced by some bacteria is possibly attributable to their lack of a vomiting mechanism. As indicated earlier, resistance to a given infectious agent is not necessarily associated with phylogenetic relationships, and there is no pattern from which the resistance or susceptibility of an animal can be predicted by logical processes; the tabulation of animals susceptible to a given disease represents information acquired largely by trial and error.

Domestic and experimental animals. Not only do species of higher organisms differ in their resistance to infectious disease but the races comprising a susceptible species likewise appear to differ among themselves. There are many instances of differences in resistance to infectious disease in varieties or strains of animals. The relative resistance of Algerian sheep to anthrax is well known, and inbred Berkshire swine have been

found to be highly resistant to brucellosis. That this is a true racial immunity which is, as might be expected, inheritable has been demonstrated by extensive experimental investigations with laboratory animals. The earlier studies of Wright and Lewis showed that marked differences in susceptibility to tuberculosis existed between inbred families of guinea pigs, differences which were transmitted to the offspring. In subsequent work[33, 35] it has been shown that resistance to infection may be raised or lowered by selective breeding, sometimes to a remarkable degree. Lurie has bred strains of rabbits resistant and susceptible to infection with tubercle bacilli. When subject to inhalation infection with human tubercle bacilli, the response of the animal is essentially all or none and sharply illustrates the role of genetic factors; in the resistant animal the bacilli multiply for only a short time and are then destroyed, while in the susceptible animal they proliferate freely for long periods.[56] It is of interest that resistance to bacterial endotoxin may also be raised or lowered by selective breeding. Resistance does not behave as a simple Mendelian character but is to some extent specific in that a race having increased resistance to infection with one microorganism is not necessarily unusually resistant to another. For example, in Webster's work mice selected for resistance to infection with *Salmonella enteritidis* showed increased resistance to pneumococcus and Friedländer's bacillus infection but were more susceptible to the virus of louping ill than the strain selected for susceptibility. In a general way, it appears that inherited resistance is, to a degree, specific in that it is effective against groups of related infections rather than against infection in general.

Considerable interest has attached to the mechanisms underlying inherited resistance and susceptibility. Thus, in Lurie's studies resistance was associated with low skin permeability as assayed by intradermal inoculation of India ink, increased rate and intensity of antibody (agglutinin) response, and the development of a high degree of hypersensitivity. It has been found that in mice there is a marked correlation between numbers of leucocytes and resistance to mouse typhoid. Similarly, resistance and susceptibility of breeds of chickens to *Sal. pullorum* infection are associated with numbers of lymphocytes; differences in liver and spleen in resistant strains of mice are associated with the ability of macrophages to digest phagocytosed bacteria, and strains of chickens genetically resistant to *Sal. gallinarum* infection have higher body temperatures than susceptible strains, and this was combined with more active phagocytosis. While such correlations as the foregoing are suggestive, in general it has not been possible to associate resistance with any single character; rather it appears to be dependent upon a complex interaction of characters that, individually, do not account for the observed resistance.[123] Differences in susceptibility may, of course, be a reflection of corresponding differences in immunizability, *i.e.*, ability to respond to antigenic stimuli with antibody production, as in Lurie's resistant rabbits, and the immune response to toxoids behaves similarly.[42]

Races of man.[90] The relative resistance of races of man to infection has been the subject of considerable interest and such investigation as has been possible. Under ordinary circumstances in this country the nonwhite races are much more susceptible to infectious disease than the white race. There are, however, certain exceptions. Thus, the influenza epidemic of 1918 appears to have had a greater impact upon the death rate for white youths than upon that for the nonwhite population in the same age group. A similar exception may be noted in the case of Baltimore Negroes who showed a lower ratio of clinical diphtheria to immunizing infections than corresponding white children. It is quite generally recognized, too, that the Negro has a remarkable degree of resistance to erysipelas, and the more favorable response of the Negro to all forms of treatment for gonorrhea is well known.

Special interest has attached to the white and nonwhite tuberculosis death rates, both crude and age-specific. Whether the observed high mortality in the nonwhite represents a racial susceptibility or is entirely a reflection of economic status has been the subject of considerable discussion.[48]

There also appear to be differences between the less well-defined "races" of man. There is evidence that the Irish are less resistant to tuberculosis than certain other elements of the American population, such as the Italians. On the other hand, the Jewish race is considered by many to be relatively resistant to tuberculosis; in spite of a high incidence of infection, the mortality is very low.[76] To what degree the evidence supports the hypothesis that races of man differ in their susceptibility to this and other diseases, such as pneumonia, is problematical, for adequate control of the environmental factors is difficult if not impossible.

In general, appropriate data on the hereditary transmission of susceptibility, or resistance, to infection in man are not available, but two kinds of evidence suggest that hereditary factors are involved. The best of such data are twin studies, monozygotic and dizygotic, the latter serving as controls. Many of these have concerned tuberculosis, and most are in agreement that there is a hereditary element in susceptibility,[96] and similar studies of susceptibility to poliomyelitis have indicated that susceptibility to the paralytic form of the disease may be conditioned by the heterozygous state of a recessive gene.[39] A hereditary element is suspected when a disease seems to "run in families." Rheumatic fever is such a disease, and the observed associations between incidence of the disease and blood relationship appear to be significant.[20, 101] The question is one of the occurrence of practically significant genetic segregation.

One of the best established cases of hereditary control of resistance to a specific infection is that of the relative insusceptibility to certain kinds of malarial infection associated with sickle-cell anemia and the Mediterranean anemia, thalassemia. These anemias are almost invariably fatal in the homozygous individual, but the individual, heterozygous for the appropriate gene, is considerably more resistant to malarial infection than the individual in whom these genes do not occur. In sickle-cell anemia hemoglobin synthesis is affected directly by the gene, and in the abnormal hemoglobin (hemoglobin S) valine is substituted for glutamic acid at one point in the peptide chain. In thalassemia the expression of the genes responsible for hemoglobin synthesis is affected quantitatively to give excessive production of the fetal type of hemoglobin (hemoglobin F). It is probable that the presence of such hemoglobins in the red blood cell makes it less susceptible to infection with malarial parasites.

A given disease, however, may be relatively mild in its effect on races of man which have been in contact with it over a long period but may assume a highly virulent form in other races to which it is new. Measles, for example, a mild disease to civilized man, has been a scourge to certain primitive races. In other cases, diseases originally highly virulent have become apparently less so with the passage of time; leprosy is not as widespread as it was in Biblical times, and syphilis is a considerably milder disease today than it was in the sixteenth century. Phenomena such as these have been taken by some to indicate the development of a racial immunity through a selection of more resistant individuals, and by others to suggest an adaptation of the microorganism accompanied by a loss of virulence. At the present time, it is not possible to differentiate sharply between these two; possibly both effects are operative.

It should be pointed out in this connection that what might be called a pseudo-racial immunity may be manifested by a race in close association with a given infective agent. Many individuals have the disease, the survivors are immune, and this immunity is passively transferred to the offspring, who are infected before this passive immunity en-

tirely disappears and consequently have the disease in a mild form but become solidly immune. The immunity is passively transferred to the third generation, and the process continues *ad infinitum* as long as the race is in contact with the disease. Some such mechanism as this appears to account for the apparent racial immunity of the West African Negro to yellow fever; the immunity of adults arises as a result of mild infection in childhood.

Age.[102] In a general way, resistance to infection increases with age, but there are a number of important exceptions. Embryonic and fetal tissues have little resistance, and susceptibility remains high for a variable, but short, time post partum. The chick embryo and associated membranes may be readily infected with a variety of agents, particularly viruses and rickettsiae, to which the chicken is, for all practical purposes, completely resistant. Similarly, on growth on culture *in vitro*, tissue explants tend to assume the physiological character of embryonic or neoplastic tissue and are susceptible to infection with a variety of agents. The persistence of susceptibility post partum is taken advantage of in the propagation of certain infectious agents, such as certain strains of poliovirus in suckling mice or the enteric infection of the suckling rabbit with the cholera vibrio.

An important factor, though not the only one, in the susceptibility of the very young to infection is the lack of an immune response. This is compensated for in the newborn mammal, so far as survival is concerned, by the presence of antibody of maternal origin (Chap. 14) which serves to provide temporary protection against the diversity of microorganisms encountered on emerging from a sterile environment. Occasionally this may not be sufficient, and fatal systemic infection with microorganisms not usually regarded as pathogenic can occur. Such infections with coliform bacilli are occasionally observed in human infants, and the disease of foals known as scours is a septicemia of coliform etiology. Apparently the immune mechanism is poorly developed even at birth, and the extremely young tissue is unable to distinguish the "not self" of foreign proteins and other antigenic substances. This has the practical consequence that prophylactic immunization of the newborn human infant is relatively ineffective.

The predominance of the diseases of childhood is not necessarily due to an unusual lack of nonspecific resistance in the pre-adolescent age groups. Man appears to be highly susceptible to diseases such as measles, mumps, and chickenpox, but the infectious agents are sufficiently prevalent in the human population that the probability of encountering them early in life is great. When the child is relatively isolated, as in certain rural areas, the probability of contracting such a disease is less, and it tends to appear later in life. It is for this reason that the so-called diseases of childhood tend to be a problem when young adults are suddenly aggregated as in military groups; such groups include a fair proportion of individuals who have, by chance, not yet contracted immunizing infections in childhood.

By puberty the average individual has acquired a variety of immunities, with or without overt evidence of infection, which are similar to the pseudo-racial immunity described above. The juxtaposition of such acquired immunities with puberty has led, from time to time, to the postulation of a "maturation immunity," a state of enhanced resistance presumed to be causally related to sexual maturity. Such an explanation for the greater resistance to diphtheria of the young adult was offered many years ago, but it was shown that this is, in fact, the result of immunizing, but inapparent, infections in childhood. Such an explanation of resistance to poliomyelitis on the part of adults was current for a time until it became clear that infection with poliovirus is very common, only the rare individual showing paralytic symptoms. Further, attempts to demonstrate a maturation immunity in experimental animals in which sexual precocity has been induced by the administration of sex hormones have failed

to provide supporting evidence. The naturally occurring association between sexual maturation and resistance to infectious disease appears to be fortuitous and not indicative of a causal relationship.

Other differences in susceptibility to infectious disease associated with age do, however, appear to have a physiological rather than an immunological basis. The two best established examples are those of the occurrence of acute fatal pulmonary tuberculosis in the young adult and the marked susceptibility of the aged to pneumococcus and similar pneumonias.

Infection with the tubercle bacillus (Chap. 31) is markedly influenced by predisposing factors, a characteristic of a well-established balance between host and parasite. In addition to factors which are grouped under the general head of stress, there appears to be a physiological susceptibility that begins to be evident in the teens and does not decline until the late twenties. This is reflected in the age distribution of death rates from this disease which, disregarding the high rate in the first year of life attributable largely to risk, reaches a peak in these age groups. The secondary peak of so-called late adult tuberculosis at around age 50 that has become apparent in the last three decades is another matter which is not relevant here. The physiological basis of heightened susceptibility to this disease of the young adult is not understood.

The greater susceptibility of the aged to pneumonias is generally regarded as an expression of an accumulation of degenerative changes which find one expression in this way. It has not been possible to associate this susceptibility with specific physiological changes, but failure to accumulate immunities to microorganisms such as pneumococcus and streptococcus during the life history of the individual, because these immunities are transient, is no doubt a factor, but hardly a differential one.

A consequence of the differences in resistance, or acquired immunity, to various pathogenic microorganisms associated with age groups is that the prevalence of disease, but not necessarily infection, is a function of the age distribution of the population (Chap. 10).

Sex. A number of diseases exhibit a difference in sex incidence; pneumonia and epidemic meningitis, for example,

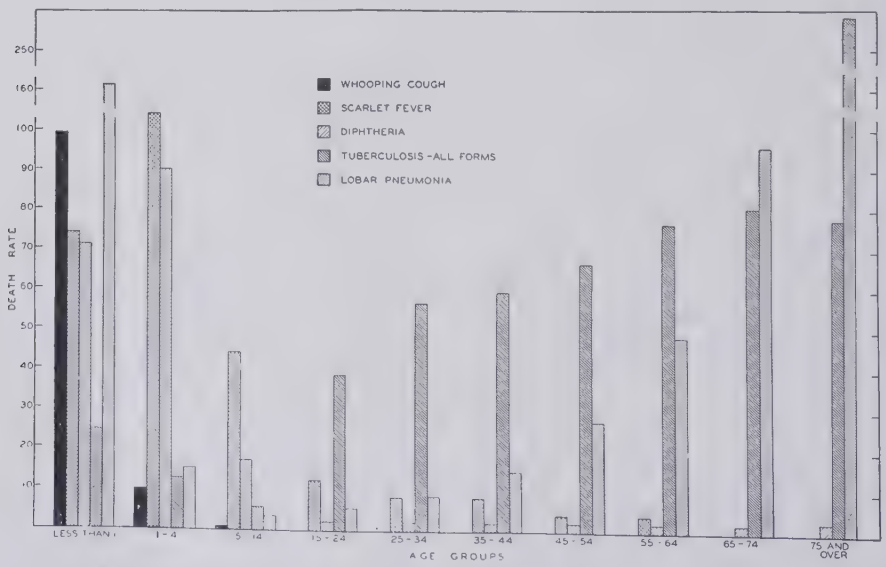


Figure 59. The age incidence of certain infectious diseases. Note the predominance of the diseases of childhood in the early years, the increase in tuberculosis in the young adult years, and the marked increase in lobar pneumonia with advancing age. Scarlet fever rate, multiplied by a factor of 40 and diphtheria rate by a factor of 10, for comparative purposes. Data from the reports of the Bureau of Census.

are more common in males than females, while scarlet fever, typhoid fever, and others occur somewhat more frequently in females. Except for a few years in the 41-year period 1900–1940, the female death rate for white youths has been consistently lower than that for males, though the reverse has been true for the nonwhite population in this country. In the decade 1931–1940 the gap between the female and male in the nonwhite population was closed, and in 1940 the female and male death rates for nonwhite youths were exactly the same at 5.0 per 1000. Corresponding rates for the white population were 1.4 for females and 2.0 for males. In the case of tuberculosis, variability in the age-specific mortality rates indicates a differential response on the part of the sexes. A sex differential is also indicated by the aftermath of the 1918 influenza epidemic, which appears to have had a more unfavorable effect upon female mortality than that of males in the 15- to 24-year age group, as indicated by the relatively high female death rate for all racial groups for several years after the epidemic.

It has been suggested that this difference between the sexes might result from resistance having some of the attributes of a sex-linked character, or, since differences in sex incidence are most pronounced during and after puberty, something in the nature of maturation immunity may be involved. There is no sound evidence to support the first of these hypotheses. Regarding the second, a certain amount of experimental evidence has been reported, the most precise relating to skin permeability, which has been found to be greater in female rabbits than in males. It has also been shown that female chicks are significantly more susceptible to infection with the chicken malaria parasite, *Plasmodium gallinaceum,* than are males; treatment with male and female sex hormones did not affect the differences between the two. Any explanation of the observed sex differences must, of course, take into consideration other factors such as occupation, risk of exposure, and the like.

Climate and Season.[61, 69, 106] That both climate and the season of the year exert marked effects on the incidence and mortality of a number of infectious diseases is well known. In tropical climates, for example, the acute upper respiratory infections are not so common as in the temperate climates, but the dysenteries are more common in the tropics than in the temperate zones. The seasonal incidence of the infectious diseases is also common knowledge; meningococcus meningitis occurs predominantly in the winter and spring, poliomyelitis in the late summer and early fall, and so on. One of the most striking relationships of season to incidence of disease is that of Asiatic cholera; the epidemic season coincides with hot weather and shows a remarkable correlation with precipitation and relative humidity.

In a great many instances the influence of climate and season upon the incidence of disease may be attributed to opportunities for transmission of the causative organism, such as crowding in poorly ventilated rooms, seasonal or geographic occurrence of an insect vector, and so on, but in others the factors responsible for seasonal incidence are unknown.

There is a good deal of evidence which substantiates the opinion that resistance varies with season, temperature, and similar factors. For example, the intensity of the brain reaction of mice inoculated with St. Louis encephalitis virus and of guinea pigs inoculated with endemic typhus rickettsiae has been found to be the greatest in summer and the least in the winter over a period of years; mice adapted to existence in a moist heat have been found only one-quarter as resistant to infection with hemolytic streptococci as those adapted to a cool environment; the morbidity and mortality rates in murine typhus infection in mice are affected by environmental temperature; and the resistance of mice to pneumococcus infection is

similarly affected. In at least some instances seasonal variation in resistance may be correlated with diet (see below). It is not improbable that future investigation will yield systematic knowledge of the effect of climatic factors on susceptibility to disease.

General Physiological Well-being. Whatever resistance to disease an organism may possess by virtue of species, race, and the like is profoundly influenced by its general physiological state. In general, resistance is at its height when the organism is functioning normally in every respect and is reduced by a variety of factors which interfere with and alter the normal physiological state. In some cases previous infection may reduce resistance to such a point that infection with the less virulent bacteria may take place, as in secondary infections; in others, functional disorders such as diabetes mellitus bring about a reduction in resistance to infection. More common, however, are the deleterious effects of inadequate diet and fatigue.

Nutrition.[2, 14, 21, 85, 86, 87, 88] The relation between susceptibility to infection and faulty nutrition has been of considerable interest in connection with the study of deficiency diseases. There is little doubt that nutritional status is of practical significance to the incidence of and mortality in infectious disease. The general problem has not responded to experimental study as readily as might be supposed. In a great many instances inanition is not clearly separable from a specific deficiency, such as a vitamin or amino acid deficiency, and results are complicated by such "side reactions" as dehydration resulting from reduced water intake by animals on certain deficient diets. In general, however, it seems to be clear that diets qualitatively and quantitatively inadequate may predispose to bacterial infection. The reduced resistance appears to be associated with a reduced humoral immune response, but this is in part at least compensated for by an accentuated phagocytic response to infection.

The importance of adequate protein intake has been emphasized by Cannon and his co-workers,[12] and they have shown that depletion of the protein reserves of experimental animals by maintenance on low protein diets interferes markedly with antibody formation, *i.e.*, the synthesis of immune globulin. While antibody formation is significantly interfered with by moderate protein depletion, a severe depletion also interferes with the normal functioning of the cellular defense mechanisms as evidenced by reduced phagocytosis.

Other than this, just how malnutrition brings about a reduction in resistance is not known; present information indicates that the effect is a general one. It is of some interest that this unfavorable effect is not limited to the undernourished individual but may be transmitted to offspring. Experimental studies have shown, for example, that the diet of mice, genetically homogeneous in resistance to an infection, affects the resistance of offspring to a greater degree than the diet of the offspring themselves. In this connection, statistical studies have suggested that the mortality rates of adult human life are in part a function of nutritive and other elements of the environment of childhood; in other words, the effect may be a delayed one, but the evidence on this point is not altogether indubitable.[13]

The marked reductions in resistance associated with inadequate diets are in no sense specific; resistance to infection in general is reduced, and there is no relation between lack of a single dietary factor and susceptibility to a particular infection. A number of attempts have been made to demonstrate such a relation; ascorbic acid, for instance, has some small capacity to neutralize diphtheria toxin, but vitamin C deficiency does not predispose to infection with the diphtheria bacillus any more than with other organisms, such as the tubercle bacillus.

The apparently contradictory observation that resistance to infection with a number of the viruses is increased by inadequate diet has been made by a number of workers.[49] That it is not com-

pletely inconsistent with the lowered resistance associated with protein depletion and other dietary deficiencies is indicated by the consideration that the viruses are obligate intracellular parasites; possibly a healthy cell is of primary importance to multiplication of the virus and the depleted cell is an unfavorable medium for development, thus masking, so to speak, the lessened immune response associated with nutritional deficiencies.

More specific evidence in this connection has been reported for certain bacterial infections. It has been pointed out elsewhere that selection of virulent variants of a challenge microorganism may occur *in vivo;* virulent threonine-requiring variants of *Sal. typhimurium* are so selected by the animal given additional threonine.[66] It has been found also that an induced variant of *Sal. typhi* having complex nutritive requirements showed at the same time reduced virulence for mice. The supposition that the animal did not supply the required nutrients in adequate amount was substantiated by the observation that full virulence was restored when the animals were given supplemental amounts of the nutrients required by the bacteria—purine, *p*-aminobenzoic acid, and aspartic acid. Similar results have been obtained with purine-requiring variants of Friedländer's bacillus, and seasonal variation in virulence could be correlated with the nutritional state of the animals.[3, 4, 10]

Fatigue. It has long been known to the clinician that bodily rest is a valuable adjunct to the treatment of disease, and there is clinical evidence which suggests that resistance to the initial infection may be reduced by excessive fatigue. Experimental evidence on this point is scanty and to some degree conflicting, but it is probable that the unfavorable effect of fatigue on normal physiological well-being is reflected to some extent in an increase in susceptibility to infection. The normal white rat, for example, is highly resistant to anthrax, but when exhausted by work in a treadmill, becomes susceptible. Latent *Sal. enteritidis* infections in the same experimental animal may be activated by fatigue to such a degree that the outcome is fatal. Similarly, studies on human beings have indicated that an individual may be rendered transiently susceptible to the common cold by fatigue.

Other mechanisms operative in the resistance associated with general physiological well-being are obscure. Studies on resistance to the common cold in population groups made by subjecting the incidence data to an analysis of variance among groups have strongly suggested that a constitutional factor, as yet undefined, is operative in the etiology of the clinical infection. There is evidence too that the capacity to maintain effective circulation and the ability to withstand the effects of sudden temperature changes are associated with resistance to experimental infection. The adverse effects of sudden changes in temperature and humidity on the organism, reflected in changes in the nasal mucosa, are, perhaps, a manifestation of temperature shock. Attempts to associate such shock, vitamin deficiencies, fatigue, and other elements of the nonspecific resistance of health with specific defense mechanisms, such as the capacity to form antibodies, have not been uniformly successful.

The External Defenses of the Organism. The cellular organization of the animal body is a closed system with respect to the outside environment, from which it is separated by the skin, mucous membranes, and intestinal mucosa. These structures, generally impermeable to particulate material of the size of bacteria, constitute the first line of defense against invading microorganisms and one that is, for the most part, highly effective. While mechanical obstruction contributes in no small part to the efficacy of these barriers, both skin and mucous membranes also play an active part in the protection of the organism against bacterial invasion.

The skin. As a rule, the unbroken skin presents a more or less impassable barrier to microorganisms. Bacteria are found normally on the skin between the

superficial horny cells, but ordinarily are not able to penetrate deep into the tissues unless favored by some cutaneous injury, such as a wound or burn. Direct penetration of the skin is, however, indicated as the initial route of infection in certain infectious diseases such as Weil's disease (leptospirosis) and possibly syphilis. Also, infection via the ducts of the sweat glands and the hair follicles is strongly suggested in other instances, notably the staphylococcal and streptococcal infections resulting in acne, impetigo, boils, etc., and has, in fact, been demonstrated experimentally with these pyogenic bacteria.

The skin seems not to be a completely inert surface on which bacteria die largely as a consequence of drying. On the contrary, healthy intact skin exerts demonstrable bactericidal activity on those microorganisms not normally present, but the activity is not as pronounced as once supposed. It is regarded by some as a consequence of the bactericidal activity of unsaturated fatty acids, especially oleic acid, present in the sebaceous secretions. This activity is inhibited by serum albumin; this may be of some practical significance as regards skin immediately surrounding a burn or other lesion producing a serous exudate. Possibly related is the fungistatic action of the free saturated aliphatic acids in the hair fat of adults. Bacteria normally present upon the skin, such as the white staphylococci, are not appreciably reduced in numbers when swabbed on the clean skin, a fact which probably accounts for their constant presence on the body.

The conjunctiva. Bacteria and dust particles settling in the eyes are removed relatively rapidly by the mechanical flushing effect of the tears. The lacrimal secretions contain the enzyme lysozyme,[84] which is also present in certain tissue extracts, is identical with the avidin of egg white, and has been prepared in crystalline form. As described elsewhere (Chap. 3), this enzyme decomposes the cell walls of certain bacteria with the formation of protoplasts, but the protoplasts are ordinarily unstable and the antibacterial effect of this activity is bacteriolytic. Relatively few microorganisms are susceptible to this activity. One, *Micrococcus lysodeikticus,* is exquisitely sensitive and is destroyed by tears in dilutions as high as 1:40,000.

The nose, nasopharynx, and respiratory tract.[5, 38, 125] Bacteria and other particulate material present in inspired air are rapidly removed by passage through the tortuous nasal passages lined with mucous membrane to whose moist surface they cling. In this way air is largely freed from bacteria in the upper respiratory passages, those that pass the larynx are caught in the bronchi, and few reach the ultimate ramifications of the bronchioles. The process is so efficient that expired air contains almost no bacteria except those that are expelled in droplets by sneezing, coughing, etc.

The moist film which covers the mucosa of the upper respiratory tract and in which bacteria removed from inspired air and those arriving via the lacrimal secretions are embedded consists of mucus, a thin, highly viscid substance that, in a sense, constitutes a continuous web or membrane overlying the surfaces within the nose, sinuses, pharynx, and esophagus. This film of mucus is in constant motion as a result of the activity of cilia which sweep the mucus and its bacterial content toward the oropharynx, where it is swallowed. The exchange of mucus is rapid: that covering the posterior two thirds of the nose is replaced every 10 or 15 minutes, while that over the anterior third is removed every hour or two. Although mucus itself has no bactericidal activity, when combined with ciliary activity it constitutes a remarkably efficient means of ridding the upper respiratory passages of bacteria. Particles above 7 μ in diameter which are inhaled are retained in the upper respiratory passages of the rabbit, about half the particles 3 μ in diameter are similarly removed, and the remainder plus practically all those 1.5 μ in diameter and smaller penetrate into the lungs (see also Chap. 10). The bacteria that penetrate the upper respiratory passages and lodge in the bronchi and

bronchioles are probably phagocytosed by the fixed alveolar epithelial cells and the wandering leucocytes that enter the bronchioles and sacs, a process discussed at greater length elsewhere.

Lysozyme is, of course, present in the nasal mucus, and it has been observed that the normal serous secretion of the nose contains a virus-inactivating agent distinct from lysozyme. The activity is virucidal for the influenza and certain other viruses inactivated by sodium desoxycholate, but what part it plays in resistance to infection is not clear.

The mouth, stomach, and intestinal tract. The mouth contains a predominant normal bacterial flora and a minor transient flora. The former consists of microorganisms which have established positions between the teeth, on dental plaques, in the crevices between the teeth and the gums, etc., while the latter represents a constant contamination. Both are subject to constant depletion because of the flushing action of the saliva. Saliva is thought by some to be mildly bactericidal possibly because of the presence of small amounts of peroxide accumulating as an end product of microbial respiration. The removal of bacteria is, however, largely a mechanical process. The microorganisms flushed to the back of the mouth meet with those from the nose and, with them, are swallowed.

Bacteria reaching the stomach are subject to the strongly acid environment of the normal gastric juice, and there is no doubt that the great majority of them are destroyed there. Some do, however, reach the intestinal tract, perhaps because they are embedded in solid particles of food and thus protected or because they are able to withstand a short exposure to the bactericidal action of the gastric secretions. Generally, very few viable bacteria are found in the stomach, but the numbers of microorganisms increase in the small intestine with the rise in pH from the duodenum to the ileum. The large intestine contains great numbers of bacteria derived not only from the upper levels of the intestinal tract but also from the multiplication of bacteria present in the intestines as normal inhabitants. As in the respiratory passages, mucus plays an important part in the mechanical removal of bacteria. Here, however, the mucus does not form a uniform coating over the intestinal mucosa but is present as a meshwork. The villi free themselves from particles by movements which bring them in contact with the mucus to which the particles, including bacteria, adhere. The mucus, with the embedded microorganisms, is rolled up into small masses and moved outward by the peristaltic movements of the bowel. Bacteria, then, which enter the mouth and upper respiratory tract are eventually extruded with the feces.

Microorganisms present in the lumen of the bowel, including those making up the normal intestinal flora, are, for all practical purposes, outside the tissues of the body. Penetration of the tissues from the bowel, as in typhoid fever, is usually via Peyer's patches into the lymphatic system and the blood stream through the thoracic duct. Ordinarily this barrier is a highly effective one, but one of the effects of whole-body ionizing irradiation is a breakdown of this barrier, followed by a generalized invasion of the tissues by bacteria from the intestine, and the immediate cause of death in radiation sickness may be fulminating bacteremia.

The genital tract. The normal genital tract is remarkably free from bacteria. The urethra in both male and female is normally sterile, a consequence, perhaps, of the flushing action of the slightly acid urine. The few bacteria that may be present are confined to the region of the meatus. The normal vaginal secretion is acid and is markedly bactericidal toward most species of bacteria.

Internal Defenses. On gaining access to the tissues, the pathogenic microorganism needs to establish and maintain foci of infection, and a number of internal factors in resistance may be distinguished. The relative susceptibility, or resistance, of tissues that finds expression as the cytotropism of microorganisms in naturally occurring infections has been described elsewhere (Chap. 4). Other

factors include the relevant physiological responses subject to hormonal regulation, and the antimicrobial activities demonstrable in tissues and organs.

Adrenocortical hormones.[45, 46, 47, 100, 112] Hormonal imbalance, and consequent disturbance of a wide variety of functions, is, in general, associated with increased susceptibility to infection. The elements of natural resistance may thus be inferred from effects which result in increased susceptibility. Such effects are not direct in that the internal secretions affect the microorganism directly, but are concerned, though not necessarily linked, with physiological patterns of response to stimuli such as those provided by the pathogenic microorganisms and their products.

The well-known predisposition of the diabetic to infection, such as gangrenous disease of the extremities, is perhaps to be associated with circulatory disturbance and illustrates the indirection of the relation of hormonal imbalance to natural resistance. Similarly, hypothyroidism results in reduced resistance to experimental tuberculosis,[54, 55] but the effect on the specific immune response is less clear and appears to vary with animal species.[91] Of the hormones, those of the adrenocortical group have been of the greatest interest because of the marked depression in resistance that they produce. In essence, this effect is a consequence of a depressing effect on the inflammatory response and the activity of the cells of the macrophage (reticulo-endothelial) system and emphasizes the significance of these cells in natural resistance. The cellular response to infection is qualitatively the same in the normal and specifically immune animal but is markedly accelerated and accentuated in the latter, and is considered elsewhere (Chap. 14) in this connection. Here the effects of this group of hormones may be regarded as both broadening and modifying the contribution of this system of cells to resistance, as well as indicating the significance of other, probably less important, factors.

The effects of administration of adrenocortical hormones on infections have been summarized by a number of reviewers. The administration of ACTH or cortisone to patients with acute febrile illness usually leads to a prompt defervescence in proportion to the severity of the symptoms, but at the same time there is not only no improvement with respect to the microorganism, but the infection may spread and secondary infections appear almost asymptomatically because of the effect of the hormone. Herpes simplex appears to be one of the exceptions, for topical application of corticosteroids, common in ophthalmology, to herpetic infections of the eye results in an exacerbation of the disease process with the spreading destructive involvement which has often resulted in perforation of the cornea.[43] Administered to normal animals, these hormones depress natural resistance to experimental infection except in those cases, such as pneumococcal infection in the mouse, where susceptibility is already maximal, and lead to the activation of latent infections if these are present. Further, when given in combination with antibiotics, the efficacy of the chemotherapeutic agents is reduced, emphasizing the significance of the role of host factors in successful chemotherapy. These hormones also accentuate the activity of endotoxins, as indicated earlier in connection with the Shwartzman phenomenon, not by affecting the toxin directly, but by interfering with the cellular response and detoxifying mechanisms. Pre-existing antibody is not affected, nor is the antigen-antibody union, but antibody formation is depressed by these hormones. It is, therefore, not surprising that the appearance of infection is a major reason for discontinuance of cortisone or ACTH therapy initiated for other purposes or that the immediate cause of death in spontaneous hyperadrenalism (Cushing's syndrome) is often infection, and the death rate from this disease has not been altered appreciably since the introduction of antimicrobial chemotherapeutic agents.

Organ and tissue factors.[41, 97] The possible presence of antimicrobial substances in various tissues and organs is

a matter of considerable interest but has not been extensively studied. Two kinds of substances, the one water- or saline-soluble and the other lipid in nature, have been prepared from a wide variety of tissues.

The water-soluble substances found in saline extracts of hog pancreas and thymus and calf thymus are basic polypeptides containing relatively large amounts of lysine or arginine. These substances have, in the aggregate, antibacterial activity on microorganisms such as the anthrax bacillus, streptococci, staphylococci, and tubercle bacilli, differing from one to another in activity. Similar polypeptides are also found in inflammatory exudates in the lung which are active against pneumococci. It is not fully established that all of these substances are not artifacts formed by, for instance, hydrolysis of histones during extraction. Spermine, found in semen and in tissues such as beef kidney, has antibacterial activity against tubercle bacilli. Substances of lipid nature, soluble in organic solvents and slightly soluble in water and saline, have also been isolated from the liver and spleen of rats and guinea pigs and have antipneumococcal activity which is directly related to the susceptibility of the animal species to infection with these bacteria. In most instances such a direct relationship between antimicrobial activity and the susceptibility of the animal from which the tissue extract was derived has not been established.

Interferon, the activity produced by tissue culture cells in response to myxoviruses, and perhaps others, and described earlier (Chap. 4), is not, like the tissue factors described above, pre-existing. While some activity is demonstrable *in vivo*, any contribution such activities may make to natural resistance is as yet uncertain.

Properdin.[70, 71, 72, 116, 118] This high molecular weight antigenic fraction of human euglobulin, representing no more than 0.03 per cent of the total serum protein, was described by Pillemer and his co-workers as having marked antimicrobial activity against gram-negative enteric bacilli and the Newcastle disease virus when combined in the properdin system consisting of properdin, all four components of complement (Chap. 13), and magnesium ions.

Properdin is inactivated by irreversible combination with a substance known as zymosan. Zymosan is the insoluble residue of yeast cells which have been digested with trypsin and extracted with water and alcohol and consists largely of carbohydrate from the yeast cell wall. The properdin-zymosan complex selectively inactivates the third component of complement (C'_3) in the presence of magnesium ions at 37° C. but not at 17° C. The activity is titrated by preparing a serum lacking in properdin but containing C'_3, by treatment with zymosan at 15 to 18° C. The unknown serum, or body fluid, etc., is then titrated for properdin by mixing varying dilutions with the properdin-free serum plus zymosan, incubating, and titrating for C'_3 in the usual hemolytic system. A unit of properdin is defined as the smallest amount which will remove, as the properdin-zymosan complex, 120 units (50 per cent) of C'_3.

The nature of properdin is obscure, i.e., whether it is a distinct molecular species having multiple properties, or an aggregate of substances is not known. Though originally described as a normal serum component associated with an antimicrobial activity distinct from that of the antibody-complement system (Chap. 14), it is now clear that serum bactericidal activity is dependent upon the presence of specific antibody occurring as "natural" antibody in "normal" serums (Chap. 15). Thus the relation between properdin and natural resistance has become quite uncertain.[63]

Cellular response.[27, 37, 80, 124] When the body tissues are penetrated by microorganisms an inflammatory reaction and characteristic cellular response occurs. The area is invaded by phagocytic cells, first heterophils or polymorphonuclear leucocytes, and then by macrophages which can develop into fibroblasts, functional in the repair process. This kind of reaction constitutes one of

the most important aspects of resistance, the inflammatory response in the nonimmune animal constituting an element of resistance. In the immune animal this response to the homologous microbial antigen is qualitatively the same, but the reaction is markedly accelerated, and is considered elsewhere (Chap. 15).

Normal Flora. A microorganism somewhat better able to resist the defensive mechanisms of the host but at the same time unable, except when resistance is reduced to a low level, to invade the body tissues, may exist in conjunction with the host as a part of the latter's "normal flora." The staphylococci which are able to resist the bactericidal action of the skin are almost invariably present on these surfaces and are regarded as "normal" inhabitants. The scanty bacterial flora of the vagina, on the other hand, is composed almost entirely of aciduric bacteria, and the bacterial types present in the intestines are determined to a considerable extent by the type of food material present, *i.e.*, the diet of the host, and by the pH of the various intestinal levels.

Certain kinds of bacteria, such as lactobacilli, spirochetes, various cocci, and the like, exist in the mouth in the interstices between the teeth and in and under tooth plaques and constitute a normal flora characteristic of this region. The bacteria commonly present in the nose and throat consists of still different forms, such as pneumococci. Friedländer's bacillus, and green and hemolytic streptococci. These organisms, while in a sense a normal flora, are not so well established as the flora of some other regions, and the nature of the microorganisms present may be determined in large part by the kind of bacteria which are constantly entering the upper respiratory tract.

As long as the resistance of the host is maintained at a sufficiently high level, the bacteria constituting the normal flora do no harm. If, however, resistance is reduced in some manner, or unbalanced by administration of antibiotics such as the tetracyclines as noted earlier (Chap. 6), the more virulent forms may invade the tissues and set up an infection. The congestion of the nasal mucosa and the consequent interference with ciliary activity and the movement of mucus, which follow the temperature shock of chilling, not infrequently make possible infection by bacteria such as hemolytic streptococci or pneumococci, which are already present.

The normal flora also functions as a part of the resistance complex, especially when the portal of entry of the pathogenic microorganism is the intestinal tract. The pathogen must compete successfully with the normal flora of the bowel to set up a focus of infection in the initiation of the disease process. The protective effect of the normal flora, which may range from competition to antagonism, may be very considerable. For example, enteric infection of experimental animals with human enteric pathogens may be difficult to establish, but when the pathogen is made drug-resistant and inoculated intragastrically with the drug, the microorganisms of the normal flora are inhibited while the drug-resistant pathogen is not and is able to establish an infection.[30]

Germ-free Animals.[68, 77, 107] The adaptation of microorganisms to a parasitic existence, indicated by the tendency of long-established diseases to become less severe and at the same time more prevalent, has been considered elsewhere (Chap. 7). The corollary is whether or not the host, through long association, becomes similarly adapted to the presence of the microorganism, even to such a degree that the relationship becomes a symbiotic one. In certain cases the presence of a parasitic microorganism bears a significant relation to the assimilation of food by the host; such, for example, is the case with respect to the leguminous plants and the root nodule bacteria, and the herbivorous animals and cellulose-decomposing microorganisms of the rumen. Whether the abundant intestinal flora of man and other animals has some analogous relation to the host has been of very considerable interest.

Nuttall and Thierfelder early showed

that guinea pigs, removed by caesarian section and kept in a sterile environment, survived for ten days or so, but Schottelius was not successful in rearing bacteriologically sterile chicks. Nutritional inadequacies of the chick diet apparently accounted for these results, for it was later shown that chicks could be raised for as long as forty days in a bacteria-free environment. Modern studies began largely with the work of Reyniers and his colleagues, and has shown that not only chickens, but also a wide variety of animals such as guinea pigs, rabbits, rats, etc., may be reared, and bred in the complete absence of bacteria. Nutritional requirements are considerably more complex than for the conventional experimental animal, and natural immunity is limited, illustrating the significant role of the active immune response in the complex commonly considered to be resistance.

REFERENCES

1. Arndt, W. F., and Ritts, R. E.: Synergism between staphylococci and Proteus in mixed infection. Proc. Soc. Exptl. Biol. Med., 1961, **108:**166–169.
2. Aycock, W. L., and Lutman, G. E.: Vitamin deficiency as an epidemiologic principle. Amer. J. Med. Sci., 1944, **208:**389–406.
3. Bacon, G. A., Burrows, T. W., and Yates, M.: The effects of biochemical mutations on the virulence of *Bacterium typhosum:* The virulence of mutants. Brit. J. Exptl. Path., 1950, **31:**714–724.
4. Bacon, G. A., Burrows, T. W., and Yates, M.: The effects of biochemical mutations on the virulence of *Bacterium typhosum:* The loss of virulence of certain mutants. Brit. J. Exptl. Path., 1950, **32:**85–96.
5. Bang, F. B.: Mucociliary function as a protective mechanism in upper respiratory tract infection. Bact. Rev., 1961, **25:**228–236.
6. Bisset, K. A.: Bacterial infection and immunity in lower vertebrates and invertebrates. J. Hyg., 1947, **45:**128–135.
7. Bliss, C. I.: The calculation of microbial assays. Bact. Rev., 1956, **20:**243–258.
8. Bliss, C. I.: Confidence limits for measuring the precision of bioassays. Biometrics, 1956, **12:**491–526.
9. Broitman, S. A., Bezman, A. L., Hazel, M. M., and Zamcheck, N.: Effect of endotoxin on gastrointestinal mucosa of the rat. Proc. Soc. Exptl. Biol. Med., 1959, **100:**557–561.
10. Burrows, T. W.: The basis of virulence for mice of *Pasteurella pestis.* Society for General Microbiology, Symposium No. 5, pp. 152–175. Cambridge University Press, 1955.
11. Burrows, W.: Endotoxins. Ann. Rev. Microbiol., 1951, **5:**181–196.
12. Cannon, P. R.: Symposia on Nutrition, vol. 2. Robert Gould Research Foundation, Cincinnati. 1950.
13. Cheeseman, E. A.: The effect of the war years upon mortality in childhood. Human Biol., 1938, **10:**537–546.
14. Clark, P. F.: Influence of nutrition in experimental infection. Ann. Rev. Microbiol., 1950, **4:**343–358.
15. Cliffton, E. E., and Cannamela, D. A.: Fibrinolytic and proteolytic activity of human 'plasmogen' prepared from fraction III of human plasma. J. Appl. Physiol., 1953, **6:**42–50.
16. Cox, H. R.: Viral and rickettsial toxins. Ann. Rev. Microbiol., 1953, **7:**197–218.
17. Cromartie, W. J., and Watson, D. W.: Toxic materials extracted from streptococcal skin lesions. Fed. Proc., 1951, **10:**352.
18. Custod, J. T., Lytle, R. I., Johnson, B. H., and Frank, P. F.: Interdependence of hyaluronic acid and M protein in streptococcal aerosol infections in mice. Proc. Soc. Exptl. Biol. Med., 1960, **103:**751–753.
19. Davies, A. M., Shilo, M., and Hestrin, S.: The influence of Aerobacter levan on the permeability of the blood vessels of the skin: Studies with antibody globulins and trypan blue. Brit. J. Exptl. Path., 1955, **36:**500–506.
20. Diamond, E. F.: Hereditary and environmental factors in the pathogenesis of rheumatic fever. Pediatrics, 1957, **19:**908–915.
21. Dubos, R., and Schaedler, R. W.: Nutrition and infection. J. Pediatrics, 1959, **55:**1–14.
22. Duran-Reynals, F.: Tissue permeability and the spreading factors in infection. A contribution to the host-parasite problem. Bact. Rev., 1942, **6:**197–252.
23. Duthie, E. S.: Evidence for two forms of staphylococcal coagulase. J. Gen. Microbiol., 1954, **10:**427–436.
24. Duthie, E. S.: The action of fibrinogen on certain pathogenic cocci. J. Gen. Microbiol., 1955, **13:**383–393.
25. Ekstedt, R. D., and Nungester, W. J.: Coagulase in reversing antibacterial activity of normal serum on *Micrococcus pyogenes.* Proc. Soc. Exptl. Biol. Med., 1955, **89:**90–94.
26. Elberg, S. S.: Factors affecting resistance to infection. Ann. Rev. Microbiol., 1956, **10:** 1–20.
27. Elberg, S. S.: Cellular immunity. Bact. Rev., 1960, **24:**67–95.
28. Enders, J. F.: A consideration of the mechanisms of resistance to viral infection based on recent studies of the agents of measles and poliomyelitis. Trans. Studies College Physicians, Philadelphia, 1960, **28:**68–79.
29. Fenner, F.: The seed and the soil: Host susceptibility in infectious diseases. Med. J. Australia, 1958, **2:**477–482.
30. Freter, R.: Experimental enteric Shigella and Vibrio infections in mice and guinea pigs. J. Exptl. Med., 1956, **104:**411–418.
31. Gale, D., and Elberg, S. S.: Studies on enhancement of bacterial infection by hog gastric mucin. J. Infect. Dis., 1952, **91:**50–62.
32. Gilbert, R. P.: Mechanisms of the hemodynamic effects of endotoxin. Physiol. Rev., 1960, **40:**245–279.
33. Gowen, J. W.: Inheritance of immunity in animals. Ann. Rev. Microbiol., 1948, **2:**215–254.
34. Gowen, J. W.: Genetic effects in nonspecific

resistance to infectious disease. Bact. Rev., 1960, **24:**192–200.
35. Gowen, J. W.: Experimental analysis of genetic determinants in resistance to infectious disease. Ann. N. Y. Acad. Sci., 1961, **91:**689–709.
36. Grant, R., and Whalen, W. J.: Latency of pyrogen fever. Appearance of fast acting pyrogen in blood of febrile animals and in plasma incubated with bacterial pyrogen. Amer. J. Physiol., 1953, **173:**47–54.
37. Harris, H.: Mobilization of defensive cells in inflammatory tissue. Bact. Rev., 1960, **24:** 3–15.
38. Hatch, T. F.: Distribution and deposition of inhaled particles in respiratory tract. Bact. Rev., 1961, **25:**237–240.
39. Herndon, C. N., and Jennings, R. G.: A twin-family study of susceptibility to poliomyelitis. Amer. J. Human Genetics, 1951, **3:**17–46.
40. van Heyningen, W. E.: Bacterial Toxins. Blackwell Scientific Publications, Oxford. 1950.
41. Hirsch, J. G.: Antimicrobial factors in tissues and phagocytic cells. Bact. Rev., 1960, **24:** 133–140.
42. Ipsen, J., Jr.: Inherent immunizability to tetanus toxoid, based on studies in pure inbred mice. J. Immunol., 1954, **72:**243–247.
43. Jawetz, E., Okumoto, M., and Sonne, M.: Studies on herpes simplex. X. The effect of corticosteroids on herpetic keratitis in the rabbit. J. Immunol., 1959, **83:**486–490.
44. Johnson, B. H., and Furrer, W.: Hyaluronic acid: A required component of beta hemolytic streptococci for infecting mice by aerosol. J. Infect. Dis., 1958, **103:**135–141.
45. Kass, E. H.: Hormones and host resistance to infection. Bact. Rev., 1960, **24:**177–185.
46. Kass, E. H., and Finland, M.: Adrenocortical hormones in infection and immunity. Ann. Rev. Microbiol., 1953, **7:**361–388.
47. Kass, E. H., and Finland, M.: Corticosteroids and infections. Adv. Intern. Med., 1958, **9:** 45–80.
48. Katz, J., and Kunofsky, S.: Environmental versus constitutional factors in the development of tuberculosis among Negroes. Amer. Rev. Respiratory Dis., 1960, **81:**17–25.
49. Kearney, E. B., et al.: Influence of varied protein intake and of tryptophane deficiency on Theiler's encephalitis in mice. J. Bact., 1948, **55:**89–111.
50. Lamanna, C.: Toxicity of bacterial exotoxins by the oral route. Science, 1960, **131:**1100.
51. Landy, M., Trapani, R. J., and Shear, M. J.: Inactivation of endotoxin by a humoral component. IV. Alteration in the immunological properties of typhoid endotoxin. J. Exptl. Med., 1959, **110:**731–750.
52. Landy, M., Trapani, R. J., and Rosen, F. S.: Inactivation of endotoxin by a humoral component. VI. Two separate systems required for viable and killed *Salmonella typhosa*. J. Clin. Investigation, 1960, **39:**352–363.
53. Larson, C. L., Ribi, E., Milner, K. C., and Lieberman, J. E.: A method for titrating endotoxic activity in the skin of rabbits. J. Exptl. Med., 1960, **111:**1–20.
54. Lurie, M. B., Zappasodi, P., Blaker, R. G., and Levy, R. S.: On the role of the thyroid in natural resistance to tuberculosis. II. The effect of hypothyroidism. The mode of action of thyroid hormones. Amer. Rev. Tuberc., 1959, **79:**180–203.
55. Lurie, M. B., Zappasodi, P., Levy, R. S., and Blaker, R. G.: On the role of the thyroid in natural resistance to tuberculosis. I. The effect of hyperthyroidism. Amer. Rev. Tuberc., 1959, **79:**152–179.
56. Lurie, M. B., Zappasodi, P., and Tickner, C.: On the nature of genetic resistance to tuberculosis in the light of the host-parasite relationships in natively resistant and susceptible rabbit. Amer. Rev. Tuberc., 1955, **72:**297–329.
57. McLeod, J. A., and McLeod, J. W.: The technique and interpretation of tests for leucocidin with special reference to the value of ethylene diamine tetra-acetic acid (EDTA). Brit. J. Exptl. Path., 1961, **42:**171–178.
58. Meyer, K.: The biological significance of hyaluronic acid and hyaluronidase. Physiol. Rev., 1947, **27:**335–359.
59. Meynell, G. G.: Inherently low precision of infectivity titrations using a quantal response. Biometrics, 1957, **13:**149–163.
60. Meynell, G. G., and Meynell, E. W.: The growth of microorganisms in vivo with particular reference to the relation between dose and latent period. J. Hyg., 1958, **56:**323–346.
61. Mills, C.: Medical Climatology. Thomas, Springfield, Illinois. 1939.
62. Moran, P. A. P.: Another test for heterogeneity of host resistance in dilution assays. J. Hyg., 1958, **56:**319–322.
63. Muschel, L. H.: The antibody-complement system and properdin. Vox Sang., 1961, **6:**385–397.
64. Nungester, W. J.: Nonspecific factors in immunity. Ann. Rev. Microbiol., 1954, **8:**363–392.
65. Oakley, C. L.: Demonstration of antigenic components in bacterial filtrates. Ann. Rev. Microbiol., 1954, **8:**411–428.
66. Page, L. A., Goodlow, R. J. L., and Braun, W.: The effect of threonine on population changes and virulence of *Salmonella typhimurium*. J. Bact., 1951, **62:**639–647.
67. Paterson, P. Y., Wisseman, C. L., and Smadel, J. E.: Studies of rickettsial toxins. I. Role of hemolysis in fatal toxemia of rabbits and rats. J. Immunol., 1954, **72:**12–23.
68. Phillips, A. W., and Smith, J. E.: Germfree animal techniques and their application. Adv. Appl. Microbiol., 1959, **1:**141–174.
69. Piccardi, G.: The Chemical Basis of Medical Climatology. Thomas, Springfield, Illinois. 1962.
70. Pillemer, L., et al.: The properdin system and immunity. I. Demonstration and isolation of a new serum protein, properdin and its role in immune phenomena. Science, 1954, **120:** 279–285.
71. Pillemer, L., et al.: The properdin system and immunity. III. The zymosan assay of properdin. J. Exptl. Med., 1956, **103:**1–13.
72. Pillemer, L., Hinz, C. F., and Wurz, L.: Preparation and properties of antihuman properdin rabbit serum. Science, 1957, **125:**1244–1245.
73. Ponder, E.: The Mammalian Red Cell and the Properties of Hemolytic Systems. Grune and Stratton, New York. 2nd ed., 1948.
74. Pope, C. G., and Stevens, M. F.: The purification of diphtheria toxin and the isolation of crystalline toxin-protein. Brit. J. Exptl. Path., 1958, **39:**139–149.
75. Pope, C. G., and Stevens, M. F.: The properties of crystalline diphtheria toxin-protein: Its

antigen composition as determined by gel-diffusion methods. Brit. J. Exptl. Path., 1958, **39:**150–157.
76. Rakower, J.: Tuberculosis among Jews. Mortality and morbidity among Jewish ethnic groups. Tuberculosis among Yemenite Jews. Etiologic factors. Amer. Rev. Tuberc., 1953, **67:**85–93.
77. Reyniers, J. A.: Micrurgical and Germ-Free Techniques: Their Application to Experimental Biology and Medicine. Thomas, Springfield, Illinois. 1943.
78. Ribi, E., Milner, K. C., and Perrine, T. D.: Endotoxic and antigenic fractions from the cell wall of *Salmonella enteritidis*. Methods for separation and some biologic activities. J. Immunol., 1959, **82:**75–84.
79. Ribi, E., et al.: Physical and chemical analysis of endotoxin from *Salmonella enteritidis*. J. Immunol., 1960, **84:**32–47.
80. Rogers, D. E.: Host mechanisms which act to remove bacteria from the blood stream. Bact. Rev., 1960, **24:**50–66.
81. Rosen, F. S.: The endotoxins of gram-negative bacteria and host resistance. New England J. Med., 1961, **264:**919–923; 980–985.
82. Rosen, F. S., and Landy, M.: Inactivation of endotoxin by a humoral component. V. EDC activity during gramnegative infection and shock. Ztschr. f. Immunitätsf. u. exper. Therap., 1959, **118:**562–568.
83. Rowsen, K. E. K.: The action of tetanus toxin in frogs. J. Gen. Microbiol., 1961, **25:**315–329.
84. Salton, M. R. J.: The properties of lysozyme and its action on microorganisms. Bact. Rev., 1957, **21:**82–99.
85. Schneider, H. A.: Nutrition and resistance to infections, the strategic situation. Vitamins and Hormones, 1946, **4:**35–71.
86. Schneider, H. A.: Genetics and nutrition of the host in relation to susceptibility to infection. Ann. N. Y. Acad. Sci., 1956, **66:**25–31.
87. Schneider, H. A.: Nutritional and genetic factors in the natural resistance of mice to Salmonella infections. Ann. N. Y. Acad. Sci., 1956, **66:**333–347.
88. Schneider, H. A.: Nutritional factors in host resistance. Bact. Rev., 1960, **24:**186–191.
89. Schwab, J. H., and Watson, D. W.: Host factors in the experimental group A streptococcal infections. The role of tissue thromboplastin. J. Infect. Dis., 1954, **95:**267–274.
90. Schweitzer, M. D.: Genetic determinants of communicable disease. Ann. N. Y. Acad. Sci., 1961, **91:**730–757.
91. Shewell, J., and Long, D. A.: The influence of the thyroid gland on antitoxin production in different species. J. Hyg., 1959, **57:**202–209.
92. Shilo, M.: Nonspecific resistance to infections. Ann. Rev. Microbiol., 1959, **13:**255–278.
93. Shilo, M., Woolman, B., and Hestrin, S.: Restriction of inflammatory response by polysaccharides. Nature, 1954, **174:**786–787.
94. Shwartzman, G.: Phenomenon of Local Tissue Reactivity and Its Immunological, Pathological and Clinical Significance. Hoeber, New York. 1937.
95. Simon, H. J.: Attenuated Infection. The Germ Theory in Contemporary Perspective. Lippincott, Philadelphia. 1960.
96. Simonds, B.: Twin research in tuberculosis. Eugenics Rev., 1957, **49:**25–32.
97. Skarnes, R. C., and Watson, D. W.: Antimicrobial factors of normal tissues and fluids. Bact. Rev., 1957, **21:**273–294.
98. Smith, T.: Parasitism and Disease. Princeton University Press, 1934.
99. Smorodintsev, A. A.: Basic mechanisms of nonspecific resistance to viruses in animals and man. Adv. Virus Res., 1960, **7:**327–376.
100. Spink, W. S.: ACTH and adrenocorticosteroids as therapeutic adjuncts in infectious diseases. New England J. Med., 1957, **257:**979–983; 1031–1035.
101. Stevenson, A. C., and Cheeseman, E. A.: Heredity and rheumatic fever. Some later information about data collected in 1950–1. Ann. Human Genetics, 1956, **21:**139–143.
102. Strehler, B. L., and Mildvan, A. S.: The general theory of mortality and aging. Science, 1960, **132:**14–21.
103. Symposium: Mechanisms of Microbial Pathogenicity. Society for General Microbiology, Symposium No. 5. Cambridge University Press, 1955.
104. Symposium: Microbial toxins. Bact. Rev., 1955, **19:**263–265.
105. Symposium: Non-specific effects of gram-negative bacteria and polysaccharide complexes on resistance to infection and on antibody formation. Fed. Proc., 1957, **16:**856–872.
106. Symposium: Symposium on weather and disease. Proc. Roy. Soc. Med., 1958, **51:**259–266.
107. Symposium: Germfree vertebrates: Present status. Ann. N. Y. Acad. Sci., 1959, **78:**1–400.
108. Symposium: Biochemical aspects of microbial pathogenicity. Ann. N. Y. Acad. Sci., 1960, **88:**1021–1318.
109. Symposium: Nonspecific resistance to infection. Bact. Rev., 1960, **24:**1–200.
110. Symposium: Genetic perspectives in disease resistance and susceptibility. Ann. N. Y. Acad. Sci., 1961, **91:**595–818.
111. Thomas, L.: The physiological disturbances produced by endotoxins. Ann. Rev. Physiol., 1954, **16:**467–490.
112. Thomas, L.: Cortisone, ACTH, and infections. Bull. N. Y. Acad. Med., 1955, **31:**485–499.
113. Thomas, L.: The role of epinephrine in the reactions produced by the endotoxins of gram-negative bacteria. J. Exptl. Med., 1956, **104:**865–880.
114. Thomas, L.: Physiologic and pathologic alterations produced by the endotoxins of gram-negative bacteria Arch. Intern. Med., 1958, **101:**452–461.
115. Thomas, L., and Good, R. A.: Studies on the generalized Shwartzman reaction. I. General observations concerning the phenomenon. J. Exptl. Med., 1952, **96:**605–624.
116. Wardlaw, A. C., and Pillemer, L.: The properdin system and immunity. V. The bactericidal activity of the properdin system. J. Exptl. Med., 1956, **103:**553–575.
117. Wattenberg, L. W., Elisberg, B. L., Wisseman, C. L., Jr., and Smadel, J. E.: Studies of rickettsial toxins. II. Altered vascular physiology in rickettsial toxemia of mice. J. Immunol., 1955, **74:**147–157.
118. Wedgwood, R. J., Ginsberg, H. S., and Pillemer, L.: The properdin system and immunity. VI. The inactivation of Newcastle disease virus by the properdin system. J. Exptl. Med., 1956. **104:**707–725.

119. Weil, M. H., and Spink, W. W.: A comparison of shock due to endotoxin with anaphylactic shock. J. Lab. Clin. Med., 1957, **50:**501–515.
120. Weil, M. H., and Spink, W. W.: The shock syndrome associated with bacteremia due to gram-negative bacilli. Arch. Intern. Med., 1958, **101:**184–193.
121. Westphal, O.: Pyrogens. In Springer (ed.): Polysaccharides in Biology; Transactions of the Second Conference, pp. 115–220. Josiah Macy Jr. Foundation, New York. 1956.
122. Westphal, O., Lüderitz, O., and Bister, F.: Über die Extraktion von Bakterien mit Phenol/Wasser. Ztschr. f. Naturforsch., 1952, **7B:**148–155.
123. Wier, J. A., Cooper, R. H., and Clark, R. D.: The nature of genetic resistance to infection in mice. Science, 1953, **117:**328–330.
124. Wood, W. B.: Phagocytosis, with particular reference to encapsulated bacteria. Bact. Rev., 1960, **24:**41–49.
125. Wright, G. W.: Structure and function of respiratory tract in relation to infection. Bact. Rev., 1961, **25:**219–227.

Chapter 10

THE EPIDEMIOLOGY OF INFECTIOUS DISEASE

WHATEVER the pathogenic powers of a microorganism and the efficiency of the defensive mechanisms of the host, an essential preliminary to the production of infectious disease is a meeting of the parasite and its prospective host. In some instances in which the bacterium is naturally saprophytic, it enters the body by accident, so to speak; such, for example, appears to be the case in tetanus, gas gangrene, and similar infections. In most instances, however, the microorganisms that produce disease are more or less closely adapted to a parasitic existence and pass from one animal body to another with only a relatively brief sojourn in the external world. In general, then, the transmission of infection is a process in which the causative microorganism is transferred, either directly or indirectly, from a diseased to a healthy susceptible animal.[39]

The elucidation of the mechanisms involved in this transfer is a matter of considerable importance. If the sequence of events that precedes infection is known, it may be, and often is, possible to interrupt it at its most vulnerable point and thereby control the spread of disease. Disease is by no means entirely a matter of host resistance and microbic virulence; it is, in a very real sense, the outcome of the interaction of the host and parasite populations. It is at this point that the study of the infectious diseases transcends the microbiology of clinical medicine, with its emphasis on the individual case, and assumes broad biological significance as a problem in interspecies competition.

The equilibrium that tends to become established between the host and parasite populations is an unstable one in that the factors which determine it—*i.e.*, the character of the host population in particular and possibly that of the parasite population, as well as the environmental factors which affect their relationship—are constantly shifting, and the equilibrium ever has a tendency to establish itself at a new level. The shift may be a sudden and violent one whose outward manifestation is an explosive outbreak of disease or, less commonly, may take the form of a gradual increase or decrease in the incidence of the disease.

The factors associated with the maintenance or shift of this equilibrium are the subject matter of *epidemiology*.[4, 7, 10, 23, 24, 34] The term epidemiology is best regarded in this broad sense and therefore includes the study of the transmission of *endemic* disease, *i.e.*, disease which has a low incidence but is constantly present in a population, as well as the study of *epidemic* disease, *i.e.*, disease of high morbidity which is only irregularly present in clinically recognizable form. In this connection it may be noted that the term *pandemic* is often applied to an epi-

demic of unusually great proportions. These categories, although useful, are not mutually exclusive; a disease endemic in a community may, at times, attain the proportions of an epidemic and later subside to an endemic level.

Essential to knowledge of the epidemiology of disease are certain characteristics of the etiological agent and of the clinical infection which determine the possible channels of transmission. The more important of these are:

(1) The route by which the infective agent enters the body
(2) the route by which the infective agent leaves the body
(3) the resistance of the microorganism to the deleterious effects of the outside environment
(4) presence or absence of an intermediate host
(5) the relation between frank, clinically recognizable disease and the discharge of virulent bacteria from the body.

These are most readily and satisfactorily determined by direct study of the etiological agent under controlled conditions as well as the pathogenesis of the naturally occurring disease. When the etiological agent is unknown, however, a first approximation of these properties may be inferred with surprising confidence from the observed epidemiological behavior of the disease. For example, although the famous Broad Street Pump epidemic occurred prior to the discovery of the etiology of cholera, the indirect evidence plainly indicated to Snow that the infective agent left the body in the feces and entered the gastrointestinal tract via the contaminated well water.[30]

The Carrier. With regard to the last characteristic listed above, additional explanation is desirable. In the early days of bacteriology it was assumed that the contact of host and parasite could have only one or the other of two outcomes; either no infection occurred, owing presumably to high resistance on the part of the host, or clinically characteristic disease developed in the individual. Subsequently it became clear that an intermediate state, the establishing of a symptomless infection, may occur. Such infections are, of course, inapparent and concealed and may be demonstrated only by isolation and identification of the infectious agent. An individual so infected is termed a carrier.

Two types of carriers are commonly differentiated, the casual carrier who harbors the microorganism temporarily, a matter of a few days or weeks, and the chronic carrier who remains infected for a relatively long time, sometimes throughout life. Such individuals serve to disseminate the infectious agent. In the first group are the great majority of carriers of the diphtheria bacillus, meningococcus, pneumococcus, certain streptococci, etc., and the second includes carriers of certain of the enteric bacilli, especially the typhoid bacillus. A third type of carrier is often differentiated, the convalescent carrier, who remains infected for a greater or lesser length of time after recovery from the disease. These last do not fall into the category of concealed infections. Sharp separation is sometimes not possible, however, for the casual or chronic carrier may, in fact, be convalescent from the disease in a form either atypical or so mild as to go unrecognized (ambulatory cases).

While it is now commonplace to recognize the existence of the carrier state, the implications of the general principle that infection may occur without disease are frequently neglected. Thus it follows that clinically apparent infections may constitute only a part, in some instances only a very small part, of the infections continually taking place. Clearly, then, if an important proportion of the infections are of the concealed type, a reasonably accurate estimate of the extent to which the infection is disseminated in the host population cannot be arrived at on the basis of cases of the disease. The implications of this are several. For example, diseases which occur sporadically and do not seem to be easily or often transmitted from the sick to the well, such as poliomyelitis, meningococcus meningitis, and pneumococcus pneumonia, may be as widely disseminated and readily communicable as measles or the common

cold, but the clinically distinctive disease is the exception rather than the rule. Furthermore, the rise or decline of infectious disease or its age or geographical distribution may not reflect a corresponding variability in the prevalence of the infection but rather may be a consequence of variation in the case-carrier ratios. Though here inferred from the observed occurrence of the carrier state, none of these possibilities remains purely hypothetical for all have been found to exist. Thus, carriers of virulent pneumococci do not occur predominantly in the higher age groups, or diphtheria bacillus carriers in the school child, where the morbidity of these diseases is highest; diphtheria bacillus carriers are as common in the tropics as in temperate climates despite the relative rarity of clinical diphtheria in the hot climates; and so on. In many other cases, such as that of poliomyelitis, such a situation is suspected but technically difficult to prove. It will be obvious, therefore, that the recognition of the carrier state and its implications is basic to sound epidemiological thinking and of primary importance to the understanding of the mechanism of spread of the infectious diseases.

EPIDEMIOLOGICAL TYPES OF INFECTIOUS DISEASE

On the basis of such fundamental information, the infectious diseases may be separated into a number of epidemiological types which, despite certain limitations, serve to illustrate the diversity of ways in which infection may be disseminated.[6] A rough classification, based on the assumption that the human being is the recipient of infection and that the control of diseases of man is the point at issue, follows:

(1) diseases of lower animals transmissible directly to man (rabies, tularemia, glanders, etc.)
(2) diseases of animals or man transmitted by insect vectors in which
  (*a*) the insect acts as a mechanical carrier *e.g.*, the house fly (typhoid fever)
  (*b*) the parasite multiplies in the insect vector (bubonic plague)
  (*c*) the parasite is transmitted from one insect generation to the next by egg infection (spotted fever)
  (*d*) the parasite undergoes a portion of its life cycle in the insect (malaria)
(3) diseases of animals or man transmitted indirectly
  (*a*) by water (the enteric infections such as typhoid fever and cholera)
  (*b*) by milk (scarlet fever, bovine tuberculosis, undulant fever, etc.)
  (*c*) by food (typhoid and paratyphoid fevers)
  (*d*) by inanimate objects or fomites, such as books and towels (scarlet fever, diphtheria, and the like)
(4) diseases of man transmitted directly
  (*a*) by infective droplets—airborne infection (the respiratory diseases and others)
  (*b*) by direct contact (the respiratory and venereal diseases in particular).

The epidemiology characteristic of a given disease shows, on the one hand, certain, and sometimes close, relationships to other similar infections, and, on the other, a certain variability which arises as a result of transmission in more than one way. The enteric infections, cholera, typhoid fever, and the bacillary dysenteries, are similar to one another in epidemiology but are quite different in this respect from the respiratory diseases or the insect-borne diseases. The epidemiology of typhoid fever, however, is variable within limits and depends to some degree upon whether the disease is water-borne, milk-borne, or transmitted by food or contact. In the case of water-borne typhoid, the drinking water supplies the connecting link, and the ensuing epidemic, usually explosive and limited to the area supplied by the contaminated water, shows no respect for age, sex, or economic status. Milk-borne typhoid, on the other hand, geographically limited by the route by which the infected milk is delivered, exhibits an increased incidence in the lower age groups and among females and is somewhat more frequent among those of higher economic status. Food-borne infection is still more limited, often within a family group, with no discrimination as to age or sex.

Variation in the epidemiological char-

acter of a disease may also occur with changes in the behavior of the host population. For example, the age incidence of poliomyelitis has altered in the past few decades, with a shift to the higher age groups and disappearance of the marked preponderance in young children, coupled with an increasing frequency of epidemic outbreaks of the disease. The shift has been greater in the North than in the South in this country, greater in rural than in urban areas, and greater in the United States than in other countries. This shift is commonly interpreted as evidence of postponement of effective contact with the virus, and there is some evidence that this is associated with economic status, *i.e.*, the shift is more marked in the higher economic brackets.[3] The shifting pattern in the behavior of poliomyelitis is more complex than this, and is discussed in more detail elsewhere (Chap. 39).

Airborne Infection.[35] Many of the diseases of man are transmitted as airborne infection in which suspended infectious material is inhaled. In contrast to the indirect transmission of disease by, for example, agencies such as water and milk, this kind of link between the source of infection and its recipient is not readily broken, and it has usually not been possible to control airborne infection in this way to any practical extent. Under natural conditions, the infectious material may occur in finely dispersed form in air originating directly from the source of infection, or it may be present in dust that, on resuspension in air, is inhaled.

Infectious clouds. It was postulated many years ago by Pflugge that diseases of the upper respiratory tract could be transmitted by droplets containing the microorganisms and expelled from the mouth and nose during coughing and sneezing. The droplets he studied were relatively large, greater than 0.1 mm. in diameter, and seeded the air for only short distances before falling to the ground. Since epidemiological considerations demanded airborne infection effective at considerable distances, Pflugge's "droplet infection" appeared relatively unimportant. In the early 1930's, however, the matter was reinvestigated, especially by Wells and his associates, and it was found that Pflugge's evidence was incomplete, in that under usual conditions of humidity droplets smaller than 0.1 mm. in diameter are rapidly reduced in size by evaporation to leave suspended nuclei containing microorganisms that may remain viable and infective for a matter of hours. The process of expulsion of such droplets in coughing, sneezing, and talking to give rise to such suspensions has been studied by Jennison[14] and is illustrated in the accompanying figure. An example of the significance of such spread is the description of "cloud babies," infants who disseminate infectious clouds of staphylococci, and play a significant part in the dissemination of infections in nurseries.[9]

Interest in inhalation infection was markedly stimulated during World War II because of the possibility that airborne infectious materials might be used as biological warfare agents,[16] and the subject has been studied in considerable detail.[28] Infectious agents may be finely dispersed in air under controlled conditions in the laboratory by means of atomizers or nebulizers, and the cloud so produced may be studied with respect to viable microorganisms, infectivity, and the like.[41] It has been found that survival of bacteria in the ambient cloud is conditioned by relative humidity and the nature of the original suspending medium. While many of the microorganisms may be killed, it is possible to generate clouds containing large numbers of viable cells. The infectivity of such clouds of pathogenic microorganisms may be tested by exposing experimental animals in appropriate chambers,[8, 13] and it has been found that infection is readily produced by inhalation of clouds of appropriate pathogens.

Assuming the presence of viable microorganisms in sufficient number in the cloud, their distribution following inhalation is determined primarily by particle size. As pointed out elsewhere (Chap. 9), the upper respiratory tract is a highly efficient mechanism for the removal of particulate matter suspended

in inhaled air through impingement of such particles on the wet mucous surfaces. In general, large particles are removed in the nasal area, while very small particles may not be retained at all, but exhaled. Eighty per cent of particles 1.8 μ in diameter, 50 per cent of 4 μ particles, and 20 per cent of 12 μ particles penetrate the nose; when the nose is by-passed by mouth breathing, 23 per cent of 1 μ particles, 38 per cent of 2 μ particles, 52 per cent of 3 μ particles, and 68 per cent of 5 μ particles are retained in the lungs.[25] Total body retention is, however, greater than indicated by examination of the respiratory tract alone. Inhaled bacteria are also present in the esophagus and stomach. By the use of radioactive phosphorus as a tracer, it has been found[11] that there is about a 30 per cent retention of 1 μ particles in the respiratory tree but a practically complete whole body retention of the theoretically inhaled dose, with the remaining 70 per cent predominantly in the gastrointestinal tract. The occurrence of inhaled material in the gastrointestinal tract is, of course, a consequence of the removal mechanism described elsewhere (Chap. 9) in which mucus

Figure 60. The atomization of mouth and nose secretions demonstrated by high speed photography. *1*, a violent sneeze in a normal subject; note the close approximation of the teeth, resulting in effective atomization. *2*, head cold sneeze; note the strings of mucus and the less effective atomization of the viscous secretions. *3*, a stifled sneeze. *4*, sneeze through a dense face mask. *5*, cough; note the smaller discharge than in the uninhibited sneeze. *6*, enunciation of the letter "f." (Jennison.)

secreted in the upper respiratory tract is swallowed, and contained bacteria are eventually excreted with the feces.

A wide variety of laboratory procedures, ranging from pipetting, decanting of supernatants after centrifugation to opening freeze-dried cultures and the operation of blenders, generate aerosols. Infectious aerosols so produced probably account for the majority of accidental laboratory infections, and their control is a matter of both proper equipment and technique.[27, 32]

Infectious dust. The source of pathogenic bacteria in dust is twofold: the settling out of droplets containing them that are too large to remain suspended in air any great length of time; and contamination of objects, especially textiles such as handkerchiefs and blankets, with infectious secretions that, after drying, become infectious dust. For example, contamination of the environment with *Streptococcus pyogenes* by infected persons has been shown to be in part a contamination of dust. Present evidence suggests that the inhalation of airborne bacteria in dust may be quantitatively more important than that of directly expelled particles, *i.e.*, dust is probably a more prolific and constant source of contamination of air. Control measures directed toward the suppression of dust, such as the oiling of blankets and floors in infectious wards in hospitals, have been more effective in the control of infection than attempts to destroy bacteria in air, as by ultraviolet irradiation.

THE MICROBIAL POPULATION

The well known variation in the severity and "contagiousness" of the infectious diseases is a consequence of corresponding variation from one species of pathogenic bacteria to another in their ability to invade the body tissues and, once established, to produce clinical disease. As indicated elsewhere (Chap. 7), a single species is potentially variable in these respects, for such variation can be induced by appropriate experimental manipulation. The possibility of such intraspecies variation in a bacterial population existing under natural conditions is one that has intrigued students of infectious disease for many years.

It is tempting to account for the genesis and rise of epidemic disease by assuming that the causative agent of a disease of endemic proportions gains in virulence by successive passage from person to person until its pathogenicity is so enhanced that an epidemic ensues. Similarly, a sojourn in a host population containing an increasingly large proportion of immunes might be expected to result in a diminution in the virulence of the microorganism and consequent subsidence of the epidemic. Furthermore, successive epidemic waves might conceivably result from periodic fluctuations in the virulence of the parasite. Unfortunately for such an explanation, there is little or no direct evidence that alterations in virulence play an important part in the evolution of single or secondary epidemic waves, and, in nature, microbial virulence appears to be a relatively stable character.

On the other hand, differences in the severity of a single disease from one epidemic to another are, in part, attributable to the existence of strains of the infectious agent which differ from one another in virulence. Benign and malignant smallpox referred to above is a case in point, and some workers believe that some strains of the diphtheria bacillus produce a more severe disease than others (Chap. 30). Although the bacillus varies from one strain to another, available evidence indicates that within a single strain virulence does not fluctuate to a demonstrable degree.

Possibly attributable in part to alterations in bacterial virulence are the changes in morbidity and mortality of some diseases such as scarlet fever, syphilis, and tuberculosis over long periods. In the case of scarlet fever the 25 years prior to 1830 was a period of very low death rates and was followed by a 40-year period of high death rates. Since then the death rate has declined, and, though the incidence remains high, the case fatality is relatively low. In other diseases only a decline has been ob-

served. Syphilis is no longer the scourge it was in the sixteenth century, and the present decline in tuberculosis began before the institution of preventive and therapeutic measures. In still other diseases, such as measles, no such long-term alterations in prevalence have been observed. Information is as yet too limited to assess these phenomena; possibly in some diseases there are long-term periodic fluctuations in bacterial virulence (this may be an artifact and represent only variations in the prevalence of virulent "epidemic" strains of the parasite), while in others an adaptive reduction in virulence or increase in resistance on the part of the host or a combination of both may play a part.

In general, it may be said that, in the short view, the bacterial population, as it exists in nature, is remarkably stable insofar as its ability to produce disease in a host population is concerned. Although the severity of a disease may, and often does, vary from one epidemic to another, variation in virulence is not an important factor in the single epidemic wave. Over long periods, however, alterations in virulence may contribute to the changes in morbidity and mortality observed in some of the infectious diseases.

THE HOST POPULATION

In contrast to the relative stability of the bacterial population, the human population is highly variable in its resistance to infection, and the variation, attributable to both intrinsic and extrinsic factors, is not infrequently of such magnitude that its consequences are of considerable practical importance.

Since a population, human or infrahuman, is composed of individual organisms, it follows that its character is determined by the nature of these individuals and their relations to one another, and its reaction to an external influence is expressed in terms of the aggregate of the reactions of its members. The response of a human population to an infectious disease is, of necessity, measured in terms that are composites of the responses of the individual members of the population—in short, by some method of counting. Such counting is, of course, the basis of statistics, and the statistical method, with its ramifications and refinements, is a powerful tool which makes possible the study of the response of human populations to disease—a response which is measured in terms of rates, ratios, life tables, and similar numerical devices.

Intrinsic Factors. Of the intrinsic factors which determine the response of a human population to an infectious disease, one of the most important is age distribution. The quantitative predominance of the lower age groups characterizing an immature population declines with population growth while the higher age groups correspondingly increase. As a consequence, the diseases of childhood and early adult life, such as diphtheria, tuberculosis, and the like, are relatively prevalent in an immature population but become progressively less so with the passage of time, while the diseases of old age increase in incidence with the maturing of the population. The frequency of an infection is expressed as a rate, either as the number of persons infected in a given unit of time or incidence, or as the total number of persons infected at any one time or prevalence. Correction of morbidity and mortality rates is a practical necessity and is made either by the use of specific rates, *i.e.*, the proportion of cases or deaths within a specified age group, or by the use of standardized rates which are not the observed rates but rather what the observed rates would be if the age distribution of the population were that of a standard or reference population.*

* Two standards have been used in Europe: one, the population of Sweden as it existed in 1890; and the other, the population of England and Wales as shown by the 1901 census. The first is standard in age distribution only, while the second is standard for both age and sex. In this country the population of the entire country is usually taken as a standard, and the populations of states or other portions of the country compared or adjusted to it.

The sex distribution of a population and its racial composition are of somewhat lesser practical significance, although in some communities in the United States the high mortality rate of the Negro has necessitated the use of race-specific mortality rates.

Extrinsic Factors. The extrinsic factors which alter the resistance of a host population to the spread of disease may exert their effects in either or both of two ways: first, by influencing the resistance of part or all of the individuals comprising the population, and, second, by influencing the relationships between individuals. Perhaps the most important factor in the first category is active individual immunity. If a sufficient portion of a population is immune to a disease as a result of artificial inoculation or recovery from an attack, the resistance of the entire group to epidemics of that disease is of a high order, a phenomenon which has been termed *herd immunity.* Other factors may reduce the resistance of the individual; in times of stress or calamity, for example, when relatively large groups are undernourished, fatigued and exposed to inclement weather, epidemic disease may spread with great rapidity.

Equally important to the resistance of a population to epidemic disease are the factors which determine the inter-relationships of its members. Crowding in large gatherings or the enforced close association arising from inadequate housing facilities obviously provides opportunity for the dissemination of respiratory and other diseases transmitted directly from man to man and, as well, certain indirectly transmitted infections, such as louse-borne typhus fever. Similarly, the spread of enteric infections is, to a large extent, dependent upon sanitary facilities and the solution of the twin problems of water supply and sewage disposal. Group practices which support a large rat population make bubonic plague a potential menace, and the presence of large numbers of mosquitoes of the appropriate species allows the wide dissemination of malaria and yellow fever. These and other factors, political, sociological, or economic in nature, obviously exert no small influence on the resistance of a population to the spread of disease.[29]

THE INTERACTION OF HOST AND PARASITE POPULATIONS

It will be clear from the foregoing discussion that the interaction of the host

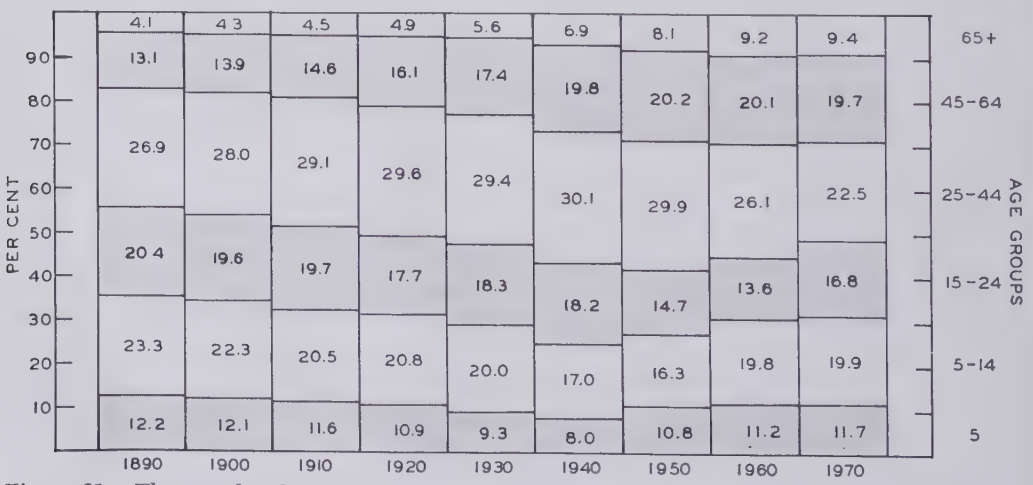


Figure 61. The age distribution of the population of the United States, enumerated 1890–1960, and projected 1970. The numbers within the columns show the per cent of the total each age group makes up in the decades shown. Data from the U. S. Bureau of the Census: Decennial Censuses, and Current Population Reports, Series P-25, No. 241, 1962. Compiled by the Population Research and Training Center of the University of Chicago.

and parasite populations is a highly complex phenomenon. Even assuming that the parasite population, when the parasite is a pathogenic bacterium, remains relatively constant in its ability to produce disease, the resistance of the host population is in a constant state of flux, and the equilibrium between the two is rarely a "steady state." As has been indicated, the relation between host and parasite populations is a part of the general problem of interspecies competition and has been studied at length. The infectious diseases of man constitute a series of special cases of the host-parasite relationship, differing from one another with respect to mode of transmission, incubation period, period of infectivity, immunity, case fatality, etc. The studies on infectious disease have taken two forms: one, the theoretical analysis of epidemic spread; and the other, the experimental investigation of controlled epidemics among populations of laboratory animals, *i.e.*, experimental epidemiology.

Theoretical Analysis.[22, 26, 31, 36] Theoretical treatment of the incidence of infectious disease in time consists of the formulation of mathematical models based upon the probability of contracting disease, independent of hypothetical variations in the virulence of the microorganism or the resistance of the host population other than as acquired immunity. A number of variables must be taken into account, including the effective contact or dose required to produce infection, the homogeneity of mixing infected and susceptible persons, infectivity of the recipient with respect to the latent or incubation period and to the onset and defervescence of the disease, and the effectiveness of the immunity associated with recovery. Of these, it is generally assumed that recovery from the disease gives an effective immunity, but the way in which the other relevant factors are presumptively defined prior to formulation in mathematical terms leads to two kinds of approaches, the one deterministic and the other stochastic.

The deterministic approach is based on the assumption that when the numbers of susceptible and infected persons are known, together with rates such as those of attack, birth, and death, any future state of the host population can be precisely determined. From the stochastic point of view, the variables are regarded as probabilities; Bartlett's formulation, for example, is based on the assumption that infection and removal of susceptibles are random events in time, and he developed the probability generating functions for these two variables—the numbers of susceptible and infected persons.

From another point of view, mathematical models are of two kinds, the point-infection type and the continuous infection type. The earlier models, including the Ross malaria equations, and the binomial expansions of Reed and Frost, of Soper, and of Greenwood, are of the point-infection type in that the period of infectiousness is contracted to a point, and the development of successive stages, or generations, of cases of disease following the introduction of infection into a susceptible population is described by the successive terms of a binomial expansion.[1] More than one probability value may be functional in a given epidemic by inhomogeneity of mixing, *e.g.*, that within families of various sizes and within the population as a whole, leading to binomial chains, and a stochastic element is introduced when the frequency distribution of these probabilities is taken into consideration.

The constant infection model of Kermack and McKendrick[15, 21] may be illustrated in deterministic form, *i.e.*, by using constant infection and removal rates. In a homogeneously mixing group of n individuals, at time t there are x susceptibles, y infectious individuals, and z who are isolated, dead, or recovered and immune; then:

$$x + y + z = n$$

If a constant infection rate is designated β, and a constant removal rate γ, the number of infections in time dt is $\beta\, xydt$,

and the number of removals is $\gamma\, ydt$, and the course of the epidemic is represented by the differential equations

$$dx/dt = -\beta xy$$

$$dy/dt = \beta xy - \gamma y$$

$$dz/dt = \gamma y$$

Approximate solution gives the rate of change of the total number of cases, or the epidemic curve, as

$$dz/dt = (\gamma^3 u^2/2\beta^2 x_0) \operatorname{sech}^2 (\tfrac{1}{2} u\gamma t - \varphi)$$

and the total size of a small epidemic is approximately

$$(2\gamma/\beta x_0)\,(x_0 - \gamma/\beta).$$

When $t = 0$ and the entire population consists of susceptibles, *i.e.*, $x = n$, unless $n > \gamma/\beta$, no epidemic can start, and the relative removal rate, $\rho = \gamma/\beta$, gives the threshold density of susceptibles. When n is greater than the threshold density, *e.g.*, $n = \rho + \nu$, the total size of an epidemic will be 2ν, reducing the initial density of susceptibles, $\rho + \nu$, to $\rho - \nu$, a value as far below the threshold ρ as it was above it initially. This is the Kermack and McKendrick Threshold Theorem and is illustrated in the accompanying figure.

When the continuous infection type of model is treated stochastically it becomes much more complex mathematically and will not be described here, but there is reason to believe that the stochastic model is a powerful tool in the study of the dissemination of infection.[5] In any case, it is clear that the spread of disease and the generation of epidemics may be accounted for by application of the principle of mass action without recourse to unfounded assumptions of systematic variation in the virulence of the microorganism.

Herd Immunity. The nature of herd immunity will become clear at this point, for the higher the proportion of immunes in a population the smaller the probability of effective contact between case and susceptible, *i.e.*, many of the contacts will be with immunes, and the population exhibits a group resistance to epidemic disease which may be of such a high order that an epidemic is no longer possible and the disease smolders in an endemic form as a result of the importation of new cases or the persistence of infection in healthy carriers whose contacts will give rise to an occasional case. A susceptible member of such an immune population, then, enjoys an immunity that is not of his own making but arises as a result of his membership in the group.

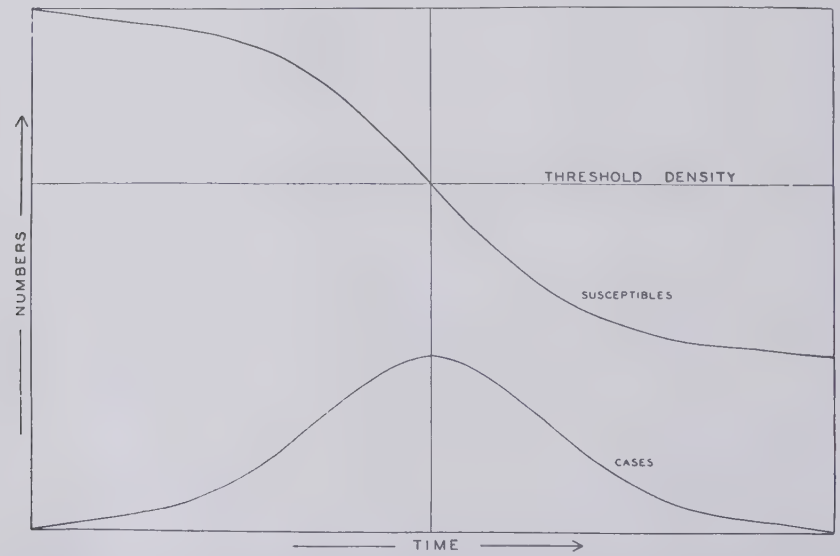


Figure 62. Diagrammatic representation of the course of an epidemic wave in terms of numbers of cases (lower curve) and numbers of susceptibles (upper curve). Note the coincidence of the peak of the epidemic wave with the threshold density of susceptibles. (After McKendrick.)

During the course of an epidemic, the decline in number of susceptibles to the threshold density coincides with the peak of the epidemic wave, and the incidence of new cases declines because the disease cannot reproduce itself. The total number of cases in the epidemic will, as described above, be twice the number of susceptibles in excess of this threshold density present at the beginning, and following subsidence of the wave the population is left with a greater or lesser degree of herd immunity.

Such precise relationships are only approximated in nature. In practice the threshold density fluctuates and is a function in part of dosage. For example, immunization of 30 per cent of preschool children suffices to control epidemic diphtheria, but the proportion is much higher in school children and 70 per cent is the commonly accepted level of herd immunity required to control epidemic diphtheria. The higher proportion required in older children is perhaps a consequence in large part of increased exposure to infection. When the prevalence of a disease is high a given individual will be subjected to a greater number of bacteria per unit time; some, previously immune to smaller doses, will become susceptibles under such circumstances and the "effective concentration," so to speak, of immunes declines with consequent effect on the herd immunity to epidemic disease. This effect of dosage on the host population has been termed *infection pressure* and is a matter of considerable practical importance. This, in effect, alters the infection rate β, taken above as invariant, since it is related to dose per unit time, and alters ρ, the threshold density. Successive epidemic waves may, then, occur as a consequence of increased infection pressure as well as through the accumulation of new susceptibles (see below). Recurrent waves of a disease that is a clinical but not an etiological entity are, of course, another matter.

Long-term Relationship. The question of the relationship between an infectious disease and a susceptible population over a long time is somewhat more complex. Mathematical treatment such as the Ross malaria equations and Martini's equations for immunizing diseases[19] is too far removed from reality to have much practical significance, principally because the mathematical approach requires that the parameters remain constant over long periods. This condition is probably rarely if ever satisfied; it is well known, for example, that within the last century both diphtheria and scarlet fever, previously of low prevalence, took on a malignant character and higher prevalence for some decades and then within the last 50 years have gradually declined in both prevalence and severity. It is of some interest, however, that the differential equations of Martini, for example, show one stable equilibrium at the origin and another at a positive value which is approached by a series of oscillations above and below the final state of equilibrium so that a series of epidemic waves appears. These solutions suggest that (a) under certain circumstances a disease may tend to die out and (b) under other circumstances a disease will reach an equilibrium after a series of epidemics of decreasing severity. Such periodicity in the incidence of many infectious diseases is well known; one of the best examples is that of measles which recurs in epidemic form at approximately two-year intervals. So far as is known, however, no infectious disease occurring under natural conditions shows a damped periodicity approaching invariancy though it has been found to occur in experimental epidemics in which the immigration rate of the new susceptibles is very high.

A disease will, of course, die out if it does not reproduce itself, *i.e.*, if the bacterium is present only in the active case and each case does not, on the average, give rise to a second case. It is not unlikely that some diseases are dying out in this way; it has been suggested, for example, that the decline in tuberculosis is due in part to inability of the disease to reproduce itself, a process accelerated by isolation of active cases. The second suggestion is of interest in connection with the epidemic periodicity of certain

diseases such as measles. It is obvious, of course, that following an epidemic a new crop of susceptibles appears, and when their numbers reach a sufficiently high level, a new epidemic ensues, and so on *ad infinitum*. It is unlikely, however, that these epidemic waves are damped as predicted by theoretical equations, for subtle variation in the complex of factors that are oversimplified as parameters will wipe out the damping effect. It will probably be many years before predictions regarding the future of infectious diseases attain a status better than that of guesses.

EXPERIMENTAL EPIDEMIOLOGY[12, 37, 38]

The information that may be derived from observation of naturally occurring disease, *i.e.*, descriptive epidemiology, is limited since the observer has no control over the process, and for all practical purposes the experiment is carried out for instead of by him. In experimental epidemics, however, the conditions may be adjusted as desired, and it may be anticipated that such experiments will be highly informative.

The study of epidemics of infectious disease developed in populations of laboratory animals under controlled conditions has been carried out by Topley, Greenwood, Wilson, and others in England and by Webster and his colleagues in the United States. These experimental epidemiological studies have been confined, for the most part, to the study of the dissemination of *Salmonella typhimurium* (*aertrycke*), *Sal. enteritidis*, *Pasteurella muriseptica*, and the virus of ectromelia (mouse pox) among populations of mice. Mouse typhoid and pasteurellosis are regarded as analogous to human diseases such as typhoid fever in which there is an imperfect immunity and carrier state, and ectromelia as analogous to human diseases in which a solid immunity is developed, such as diphtheria. The extent to which the analogies may be carried, and to which the conclusions reached are applicable to the considerably more complex human population as it exists under natural conditions, is open to some question; nevertheless, these studies, as yet in their infancy, have yielded valuable information.

The experiments which have been carried out were of two general types. The closed epidemic was produced in a population of mice, often about fifty in number, by the introduction of infected animals. The other type of experiment was carried out in an infected mouse population recruited by continuous immigration, *i.e.*, mice were added at regular intervals, the rate varying from one mouse every 3 days to six mice per day. The results may be summarized briefly:

(1) The epidemic wave initiated in the closed population by the introduction of infected individuals closely resembled those observed in the human population.

(2) The effects of dispersal of the infected population of the closed epidemic into large or small groups at various times during the development of the epidemic were studied. The time at which the population was dispersed was found to be of primary importance; the later dispersal was effected, the less favorable the result, and after the peak of the epidemic wave was reached it continued unchecked even though the population was dispersed to individual mice. This is of particular interest since fleeing from an epidemic has been a popular method of escaping infection (cf. "The Decameron"). In this connection the results of dispersal of children from the industrial centers of England in 1939–1940 may be noted. A sharp reduction of 40 per cent or more in diphtheria morbidity occurred in the evacuated towns together with an increase of 60 to 70 per cent among local children in reception centers, the latter returning to normal within six months. Similarly, the biennial periodicity of measles was broken by this dispersal.

(3) The effect of active immunization on the epidemic was not so great as

might be supposed. If the mice were immunized prior to the epidemic, a favorable effect was apparent and more marked in ectromelia than in *Sal. typhimurium* infections. Immunization undertaken after the initiation of the epidemic was without effect.

(4) In the mouse population recruited by continuous immigration a series of epidemic waves developed, the frequency of which was directly related to the rate of immigration. Thus, with a low rate, such as one mouse every 3 days, epidemic waves were separated by periods of remission in which no deaths occurred. As the immigration rate was increased, the waves occurred with greater frequency, and with the addition of six mice per day there were no periods of complete remission, the epidemic waves taking the form of periods of increased mortality.

(5) With high immigration rates, the peaks and troughs in the daily death rate became less and less pronounced after a year or more, both the rate and total population tending to become invariant. Thus it was possible to approximate experimentally the damped periodicity and eventual equilibrium predicted on theoretical mathematical grounds (see above), and the experimental host population could be brought into approximate equilibrium with the parasite population.

(6) An inadvertent experiment with a population recruited by a high rate of immigration proved to be of considerable interest. The mouse population had reached the equilibrium indicated above by summer when there was a heat wave of unusual intensity in London. There were many nonspecific deaths (mice are highly susceptible to heat), followed by violently fluctuating specific mortality. In the course of time an equilibrium was again reached but at a considerably higher death rate and decreased numbers than formerly. The parasite was shown to be unaltered in virulence, and the immigrants had not, of course, had the heat experience. Hence the host-parasite relationship *per se* was altered by an environmental "catastrophe." The relationship of these observations to the long-term relation between the human population and infectious disease is of interest. If it be assumed that the day of the mouse is equivalent to 30 days in the life of man, over the four years taken by this experiment the following were observed: (a) a period of stability with low regular death rates and growing population (*ca.* 600 days) equivalent to a human experience of slightly less than 50 years—this is longer than the 25 year period ending about 1830 during which the death rate from scarlet fever was relatively low; (b) a period equivalent to 15 years of severe and repeated epidemic waves; and (c) a period equivalent to some 30 years of relatively high mortality in a more or less stable state of equilibrium in a population reduced in numbers—the post-1830 epoch of virulent scarlet fever lasted more than 40 years.

(7) The mortality rates of the mice were intimately related to the length of time that they were members of the infected population; the rate rose rapidly to a peak in the early days of cage age and, although it declined slowly with the passage of time, to a greater extent in ectromelia than in mouse typhoid or pasteurellosis, the survivors of one epidemic wave may be the victims of another. The removal at regular intervals of a number of mice equal to the number added at the same intervals markedly altered the trend of these mortality rates; under these circumstances, the initial peak of mortality was lower, but the rapid decline was not apparent and the mortality rate remained at a high level. Removal was of considerable advantage to the individual mouse; in general, the earlier the removal the greater the advantage, except during the decline of an epidemic wave, when isolation did not increase the chances for survival of individual animals.

Although the evolution of the single epidemic wave in the experimental studies closely resembles its counterpart in the human population, the inability to control experimental epidemics by immunization is at variance with observa-

tions on epidemic disease in human populations. Some human diseases, such as diphtheria and smallpox, can be restrained from assuming epidemic proportions by active immunization of a sufficiently large number of the individuals making up the population. Whether or not this and certain other minor discrepancies prove to be real or illusory with future work, a number of pertinent suggestions have come out of these investigations. The response of the experimental host population in terms of mortality rates and cage life expectation cannot be interpreted as yet in some cases; in others the interpretation is questionable.

Some of the experiments, however, have yielded clear-cut results that bear directly upon the interaction of host and parasite populations in nature. Perhaps the most important of these is the experimental demonstration of recurring epidemic waves resulting from the continuous addition of new susceptibles to the infected population. In spite of the fact that such a sequence of events is predicted by theoretical epidemiology, as indicated above, the repeated flaring up of a disease of man or domestic animals, thought to be stamped out in the interepidemic periods, is most often taken as evidence of the re-importation of infection. The experimental demonstration of this phenomenon and the indicated futility of quarantine in the control of a disease which has become widely disseminated (only when a disease is not present, or present in limited effective foci, can quarantine and isolation procedures be effective) are, clearly, of considerable significance.

EPIDEMIOLOGICAL DATA AND THEIR INTERPRETATION[20]

The purposes of epidemiological study are threefold: first, to indicate the nature of the infective agent, its source, and modes of transmission when these are not fully established otherwise, *i.e.*, on an experimental basis; second, to extend this information into a corresponding general theory of the epidemiology of the disease; and third, to determine in detail the local conditions which favor or control the dissemination of the infection in a given area or community. For these purposes four general types of information, usually simple in character but wide in extent, are required.

Geographical Distribution. The area in which the disease occurs and the regularity of its geographical distribution are highly informative. Thus, the general occurrence of a disease indicates that environmental conditions, including fauna, climate, etc., peculiar only to parts of the area are not essential to its transmission. Similarly, restriction of the disease within geographical limits indicates that special environmental conditions are necessary for its dissemination; these may include crowding, presence of an insect vector, water supplies, proximity to reservoirs of infection, etc. In general, a uniform distribution of the disease is indicative of a simple method of transmission, as in the case of measles, whereas an irregular distribution, such as that of spotted fever, implies a more complex process dependent upon a source of infection or conditions necessary to transmission that are correspondingly irregularly distributed.

Prevalence of Disease. The prevalence of the disease suggests the source of infection. A high rate, such as that of measles, indicates that the observed cases are the most important, if not the only, source of infection. Conversely, sporadic distribution of widely separated cases implies the existence of a concealed reservoir of infection such as the casual or chronic carrier or some of the lower animals.

Seasonal Distribution. The seasonal distribution is also informative when considered together with other epidemiological features of the disease. Thus, a seasonal occurrence may be consistent with hypothesized insect transmission, or it may eliminate an insect vector as the sole transmitting agent.

Age Distribution. The age distribution of the disease is suggestive as a corollary to other epidemiological characteristics, and often aids in their interpretation. Thus, a disease occurring for the most part in the early years of life, such as measles or diphtheria, often shows differences in age incidence between urban and rural areas which are explained by the more frequent occurrence of immunizing infections, apparent or subclinical, in the more crowded areas. Since similar differentials are apparent in poliomyelitis, the age distribution of this disease, together with other of its epidemiological features, supports the view that this disease is widely prevalent as a subclinical infection.

Epidemiological evidence, therefore, consists of a series of inter-related facts from which a conclusion or series of conclusions may be drawn. The first step in epidemiological procedure is necessarily the demonstration of associations between the frequency of occurrence of the disease and some conditions or set of conditions; the second, the ascertaining of the relationship of these associations with one another; and third, their relations to the general epidemiological theory of the disease. To take a simple example, in milk-borne typhoid fever it may be ascertained that cases of the disease occur predominantly along the route of the milkman, that in families so supplied the cases occur among those who drink milk, that the cases appear within a limited time to suggest simultaneous infection, and that an employee of the dairy is a carrier of typhoid bacilli. Not only are these associations related to one another but they are consistent with the general epidemiological theory of typhoid fever, *viz.*, that transmission is basically a matter of the transfer of infected fecal material (or urine) from a patient or carrier to the mouth of a susceptible person.

While this would appear self-evident, there is an opinion of some prevalence that since epidemiological evidence is necessarily purely circumstantial, it cannot be conclusive. This is perhaps due in large part to failure to appreciate the development, often basically statistical and therefore mathematical, of the logical analysis, and the significance of the body of evidence as a whole. The method of analysis and interpretation of epidemiological evidence is identical with that of experimental evidence, the practical difference lying in the fact that, as pointed out earlier, the epidemic is an experiment done for, rather than by, the observer and cannot be manipulated.

THE CONTROL OF INFECTIOUS DISEASE[2, 40]

With an understanding, albeit admittedly imperfect, of the factors which determine the means and extent of the dissemination of infectious disease, comes the possibility of control. The variety of epidemiological types of disease and the individual variability within these broad groups make for differences in control measures, and the control of a given disease under given circumstances almost always constitutes a special case. In general, however, control measures are of three kinds, *viz.*:

(1) those directed toward reduction or elimination of the source of infection, such as
  (*a*) quarantine and isolation of cases and carriers
  (*b*) destruction of an animal reservoir of infection
  (*c*) treatment of sewage to reduce water contamination
  (*d*) therapy that reduces or eliminates infectivity of the individual
(2) those designed to break the connection between the source of infection and susceptible persons, *i.e.*, general sanitation measures such as
  (*a*) chlorination of water supplies
  (*b*) pasteurization of milk
  (*c*) supervision and inspection of foods and food handlers
  (*d*) destruction of insect vectors
(3) those that eliminate the susceptible and raise the general level of herd immunity by immunization, including
  (*a*) passive immunization to give a temporary immunity following exposure or when a disease threatens to take epidemic form

(*b*) active immunization to protect the individual from disease and the host population from epidemic disease.

One or another, or a combination of, such control measures are applicable to most infectious diseases of man and may be illustrated for the major epidemiological types of disease.

Diseases of Lower Animals Transmissible to Man.[33] Diseases in this group have in common an animal reservoir of infection. Direct control of the source of infection is, therefore, sometimes possible. When the transmission is direct, as in rabies, the reservoir of infection in the domestic dog may be controlled by immunization and quarantine of animals. The reservoir in wild animals, however, is not subject to such control, and complete elimination of the disease cannot be accomplished except locally under exceptionally favorable circumstances. The animal reservoir of bovine tuberculosis is, of course, subject to direct control and may be, and has been in this country, eliminated by slaughter of infected animals. Here the pasteurization of milk also provides protection to the human population.

Insect-borne Diseases. Control of diseases transmitted by insect vectors is often most readily approached through control of the vector. Thus malaria control is, for all practical purposes, mosquito control, and louse-borne typhus may be controlled by measures directed against the human body louse as well as by immunization of man. In instances such as these the vector is closely tied to man, by preference or by necessity, but when a disease has a reservoir of infection in wild animals and is transmitted by a vector that bites both those animals and man, control is more difficult. Plague is an example of an intermediate disease in this respect. The rat reservoir of infection is closely associated with man, and both the rat and rat flea populations are subject to an effective degree of control, but neither the wild rodent reservoir nor the insect vectors of sylvatic plague are subject to any appreciable degree of control. Similarly, tularemia and spotted fever, the latter having a reservoir in the tick, cannot be effectively controlled.

Diseases Transmitted from Man to Man. Control of the source of infection in these diseases is a matter of quarantine and isolation, and in some instances the continued application of these may tend to eliminate the source. Thus, it is possible that human tuberculosis may eventually be practically eliminated in this country by isolation of cases; for many years the disease has not been reproducing itself, and the incidence shows a steady decline. Isolation is not, however, applicable to those diseases in which the reservoir of infection is the asymptomatic casual carrier, such as diphtheria. The diseases that are largely transmitted indirectly by water, food, milk, etc., are controlled with relative facility by general sanitation practices such as chlorination of water supplies and pasteurization of milk. In some cases these control measures also eliminate or reduce the reservoir of infection in a given area. Asiatic cholera, for example, has for many years been nonexistent in this country and western Europe; it is susceptible to such elimination because no chronic carrier state occurs to maintain the disease in endemic form. Typhoid fever is a more difficult matter because of the chronic carrier state, but when it is adequately controlled, the carrier incidence declines, thus reducing the reservoir of infection. As yet airborne infection cannot be controlled so far as transmission is concerned.[17, 18]

When immunization is effective, these strictly human diseases can be controlled by its mass application, but mass immunization is weighed against other methods of control in terms of practicality. While, for example, a reasonable degree of immunity against typhoid fever may be obtained, chlorination of water supplies is a much more practical method of control of this disease than mass immunization under ordinary conditions in this country. In other diseases, however, such as diphtheria and smallpox, mass immunization is by far the most practical method of control.

REFERENCES

1. Abbey, H.: An examination of the Reed-Frost theory of epidemics. Human Biol., 1952, **24:** 201–233.
2. American Public Health Association: Control of Communicable Disease. American Public Health Association, New York. 9th ed., 1960.
3. Ames, W. R.: Variations in the age selection of poliomyelitis associated with differences in economic status in Buffalo, N. Y., 1929, 1939, 1944 and 1949. Amer. J. Pub. Health, 1951, **41:**388–395.
4. Aycock, W. L., and Russell, F. F.: The functions of hypotheses in epidemiology. Amer. J. Med. Sci., 1943, **206:**399–408.
5. Bailey, N. T. J.: Some problems in the statistical analysis of epidemic data. J. Roy. Statist. Soc., Ser. B, 1955, **17:**35–58.
6. Baker, A. C.: The typical epidemic series. Amer. J. Trop. Med., 1943, **23:**559–566.
7. Clark, E. G.: Modern concepts of epidemiology. J. Chronic Dis., 1955, **2:**593–596.
8. Druett, H. A., and May, K. R.: A wind tunnel for the study of airborne infections. J. Hyg., 1952, **50:**69–81.
9. Eichenwald, H. F., Kotsevalov, O., and Fasso, L. A.: The "cloud baby": An example of bacterial-virus interaction. Amer. J. Dis. Children, 1960, **100:**161–173.
10. Frost, W. H.: Epidemiology. In Collected Papers, pp. 493–542. Commonwealth Fund, New York. 1941.
11. Goldberg, L. J., and Leif, W. R.: The use of radioactive isotope in determining the retention and initial distribution of airborne bacteria in the mouse. Science, 1950, **112:**299–300.
12. Greenwood, M., Hill, A. B., Topley, W. W. C., and Wilson, J.: Experimental epidemiology. Medical Research Council (Great Britain) Special Rept. Ser. No. 209. 1936.
13. Henderson, D. W.: An apparatus for the study of airborne infection. J. Hyg., 1952, **50:**53–68.
14. Jennison, M. W.: Atomizing of mouth and nose secretions into the air as revealed by high speed photography. In Aerobiology, pp. 106–128. Amer. Assn. Advancement Sci., Publication No. 17, 1942.
15. Kermack, W. O., and McKendrick, A. G.: Contributions to the mathematical theory of epidemics. V. Analysis of experimental epidemics of mouse typhoid, a bacterial disease conferring incomplete immunity. J. Hyg., 1939, **39:**271–288.
16. Langmuir, A. D.: The potentialities of biological warfare against man. An epidemiological appraisal. Pub. Health Repts., 1951, **66:**387–399.
17. Langmuir, A. D.: Epidemiology of airborne infection. Bact. Rev., 1961, **25:**173–181.
18. Langmuir, A. D.: Public health implications of airborne infection: Medical aspects. Bact. Rev., 1961, **25:**356–358.
19. Lotka, A. J.: Elements of Physical Biology. Williams and Wilkins, Baltimore. 1925. Reissued as Elements of Mathematical Biology. Dover Publications, New York. 1956.
20. MacMahon, B., Pugh, T. F., and Ipsen, J.: Epidemiologic Methods. Little, Brown and Company, Boston. 1960.
21. McKendrick, A. G.: The dynamics of crowd infection. Edinburgh Med. J., 1940, **47:**117–136.
22. Muench, H.: Catalytic Models in Epidemiology. Harvard University Press, 1959.
23. Paul, J. R.: Clinical Epidemiology. University of Chicago Press, 1958.
24. Pemberton, J., and Willard, H.: Recent Studies in Epidemiology. Blackwell, Oxford. 1958.
25. Perkins, W. A., and Vaughan, L. M.: Public health implications of airborne infection: Physical aspects. Bact. Rev., 1961, **25:**347–355.
26. Raettig, H.: Die Seuchenkurve. Methoden der Darstellung und Probleme der Ausdeutung. Arch. f. Hyg. u. Bakt., 1960, **144:**159–206.
27. Reitman, M., and Wedum, A. G.: Microbiological safety. Pub. Health Repts., 1956, **71:** 659–665.
28. Rosebury, T.: Experimental Airborne Infection. Williams and Wilkins, Baltimore. 1947.
29. Sigerist, H. E.: Civilization and Disease. Cornell University Press, 1943.
30. Snow, J.: Snow on Cholera. Commonwealth Fund, New York. 1936.
31. Spicer, C. C., and Lipton, S.: Numerical studies on some contagious distributions. J. Hyg., 1958, **56:**516–522.
32. Sulkin, S. E.: Laboratory-acquired infections. Bact. Rev., 1961, **25:**203–209.
33. Symposium: Animal disease and human health. Ann. N. Y. Acad. Sci., 1958, **70:**277–762.
34. Symposium: Perspectives in epidemiology. Yale J. Biol. Med., 1959, **32:**1–61.
35. Symposium: Conference on airborne infection. Bact. Rev., 1961, **25:**173–377.
36. Taylor, W. F.: Some Monte Carlo methods applied to an epidemic of acute respiratory disease. Human Biol., 1958, **30:**185–200.
37. Topley, W. W. C.: The biology of epidemics. Proc. Roy. Soc., Ser. B, 1942, **130:**337–359.
38. Webster, L. T.: Experimental epidemiology. Medicine, 1946, **25:**77–109.
39. Williams, R. E. O.: Intramural spread of bacteria and viruses in human populations. Ann. Rev. Microbiol., 1960, **14:**43–64.
40. World Health Organization: International sanitary regulations adopted by the Fourth World Health Assembly in 1951 and amended by the Eighth and Ninth World Health Assemblies in 1955 and 1956. World Health Organization, Palais de Nations, Geneva. 1957.
41. Zentner, R. J.: Techniques of aerosol formation. Bact. Rev., 1961, **25:**188–193.

Chapter 11

THE MICROBIOLOGY OF WATER AND SEWAGE

Of the interrelationships between the members of human populations that facilitate the transmission of infection, those arising as a consequence of common water supplies and the group disposal of sewage are among the most important. Transmission of disease by this means is, of course, confined to diseases of the so-called enteric group, in which infection takes place via the gastrointestinal tract and the causative microorganisms are discharged with the feces. Infection is, therefore, the result of a direct connection between infectious fecal material and the mouth of a susceptible person, and when that connecting link is a common water supply, as it frequently is, outbreaks of cholera, typhoid fever, and the like occur. This link is, however, readily broken; the great water-borne epidemics are rapidly becoming a thing of the past as a consequence of the utilization of effective control measures, and present-day water-borne epidemics are indicative, not of a lack, but of a failure to make use, of existing knowledge.

WATER[8, 19]

Because of water-borne disease and the obvious desirability of its control, studies of the bacteriology of water have been directed, for the most part, toward its sanitary aspects. The best single criterion by which the sanitary quality of a water may be judged is, clearly, the kind and numbers of bacteria that are present in it. If it were possible, as a routine procedure, invariably to detect the presence of the appropriate disease-producing bacteria, it would be unnecessary, from the sanitary point of view, to take the nonpathogenic forms into consideration. This is, however, not the case; a judgment of the sanitary quality of a water cannot be made on the basis of failure to find a microorganism such as the typhoid bacillus in that water. This bacterium, for example, is usually so outnumbered by similar forms such as the colon bacillus that its isolation and identification require enrichment cultures in selective mediums and other time-consuming procedures; even then these are not always successful, and a negative finding is of doubtful value.

It is legitimate to assume, however, that when a water is polluted with human fecal material, it is probable that it contains bacteria which cause enteric infection. This probability becomes a certainty when the fecal material is pooled, as in the sewage of a community, because of the ubiquitous presence of healthy carriers of typhoid bacilli and similar microorganisms. Since it is impractical to isolate these pathogenic bac-

teria themselves as a routine measure, some indicator of fecal pollution will serve equally well as a criterion of the sanitary quality of a water. The question, which was stated many years ago, becomes this: Are there types of bacteria which are never present in natural waters free of pathogenic bacteria but which may always be found in water polluted with human fecal material and therefore probably with pathogenic bacteria? If so, such types of bacteria can be used as indicators of pollution. The answer to this question involves a consideration of the bacterial flora of natural waters, including both bacteria which are native inhabitants of water and those microorganisms whose presence is a consequence of contamination from external sources.

Bacteria Native to Natural Waters. The bacteria whose native habitat is water are not well known, in part because many of them are difficult to grow on laboratory mediums. There is no question, however, of the existence of a bacterial flora normal to and characteristic of natural waters. The types of bacteria which make up this natural population may be considered briefly:

(1) higher bacteria, frequently the sheathed forms assigned to the Chlamydobacteriales and including sulfur, iron, and other forms
(2) the Caulobacteria, a group of "stem" bacteria which occur in lakes and other bodies of water attached to some inanimate object
(3) the spiral forms, which are frequently found in great numbers in water, some of which may be very large, 20 to 30 μ in length, as compared with the parasitic spirilla
(4) a variety of bacilli, including
  (*a*) pigmented forms such as *Serratia marcescens* (*Chromobacter prodigiosum*), *Chromobacter violaceum, Bact. aurescens,* and others
  (*b*) various nonpigmented forms such as:
    1. the fluorescent bacteria—*Pseudomonas fluorescens*
    2. certain of the sulfur bacteria
    3. thermophiles
    4. aerobic, spore-forming bacilli of uncertain taxonomic position
(5) coccus forms, both
  (*a*) pigmented, generally yellow—very often *Sarcina lutea,* and
  (*b*) nonpigmented—*Micrococcus aquatilis, M. candicans,* and others
(6) nitrogen-fixing bacteria — *Azotobacter aquatile* in particular
(7) nitrifying bacteria—both Nitrosomonas and Nitrobacter.

These water bacteria are found in fresh water in swamps, streams, and lakes. The bacterial populations of salt waters have not been studied as extensively, but it appears[29] that the sea contains similar bacteria, including the nitrogen-fixing forms, the fluorescent bacteria, various pigmented forms, and the like. The water bacteria bear the same relation to the constant and recurring transformations of organic matter that take place in natural waters as they do to the same processes in the soil and are, therefore, an integral part of aquatic life.[7]

Some, but by no means all, of the water bacteria can be cultivated on laboratory mediums. In any case no one medium suffices; the nitrifying bacteria, for example, cannot be grown in the presence of organic matter. Since the bacteria found on plates are of necessity only those that will grow upon the medium used, it will be clear that the value of plate counts as estimates of the numbers of these microorganisms is questionable. Enrichment cultures are sometimes useful, and it may be noted parenthetically that the simple holding of a water sample for a period of hours will frequently result in a marked increase in numbers. Considerable information has been obtained by a method of "slide culture" in which a clean glass slide is suspended in the water for several days, removed, stained, and studied microscopically.

Bacterial Contamination of Natural Waters. In addition to the native water bacteria, a given water may, and usually does, contain a variety of bacteria as contamination from external sources. These sources are two—the air and soil, and human and animal excreta.

Bacteria from the air and soil. The number of bacteria in the air bears, as might be supposed, a close relation to the quantity of larger suspended particles or "dust." There are fewer bacteria in the air of the country than of the city, fewer in mountain air

than in the air of the lowlands, and the air in mid-ocean is nearly bacteria-free. Under appropriate circumstances bacteria suspended in the air are those expelled from the upper respiratory tract of human beings and are of no small significance in the transmission of "droplet infection," but insofar as air in general is concerned this is an insignificant and relatively rare occurrence. Pathogenic microorganisms, such as the tubercle bacillus and the pyogenic cocci, have been found in the air of hospitals and sickrooms, but as a rule pathogenic bacteria in dry dust are exceedingly rare. The kinds of microorganisms in the air vary somewhat in different localities, but certain forms are generally present. Molds and yeasts are quite common and in some instances outnumber the bacteria. These include various species of molds such as *Penicillium glaucum* (the blue-green mold) and various yeasts, many of them the pigmented (red) torulae. *Bacillus subtilis* and related forms, together with the various micrococci, often pigmented, are almost universally present. They find their way into natural waters through settling out or are swept down by rain.

Many of the bacteria present in the air, particularly the aerobic spore-formers, are essentially soil forms blown up in dust and able to survive drying. The soil itself contains tremendous numbers of bacteria; a gram of average field soil probably contains 100 million to 50 billion living bacteria, most of which are found in the upper 6 inches of soil, few being found in undisturbed soil below a depth of 4 to 5 feet. The great majority of these microorganisms are native to the soil and include the nitrogen-fixing and nitrifying bacteria, bacteria of the amylobacter group, and a variety of forms whose biochemical activities are an integral part or the mechanism of the decomposition of organic matter in the soil. A small part of the native bacterial flora of the soil consists of potential pathogens such as *Clostridium tetani*, *Cl. edematis*, *Cl. botulinum*, and others.

Bacteria other than those which make up the normal flora of the soil may be present as contamination. For practical purposes the pathogens that may be present may be regarded as coming from one of two sources: the flesh of animals and of persons who have died of infectious disease; and the excreta of human beings. In the first instance only one organism, the anthrax bacillus, is of significance, for other bacteria causing diseases of animals and man, such as the causative agents of tularemia, plague, spotted fever, and other diseases of animals, together with the bacteria of human disease such as the diphtheria bacillus and streptococci, do not survive long in the soil.

In the case of the anthrax bacillus, however, the spores are able to survive for long periods, perhaps years, and are brought to the surface from buried bodies by earthworms. Pastures may, then, remain infective for cattle for a long time.

The pathogenic bacteria contained in human excreta are, of course, those microorganisms which leave the body via the intestinal tract, *i.e.*, those causing the enteric diseases. The bacteria do not multiply in the soil, but their vitality may be considerably prolonged, possibly for two or three months.

It will be clear that microorganisms present in the air and in the soil have relatively ready access to bodies of water, and contamination may take place either more or less continuously or at irregular intervals under certain unusual conditions, as during and immediately after heavy rains. The contribution of air and soil, the latter in particular, to the bacterial flora of water is, then, considerable. Some of the potential pathogens, *e.g.*, the bacilli of tetanus and malignant edema, are not infective when taken by mouth, and others, even though they survive for sufficient periods, rarely produce infection by this route. The normal portal of entry of the enteric bacteria is, however, the gastrointestinal tract, and it is these microorganisms which constitute the significant contribution of contamination through the agency of soil.

The intestinal microorganisms may be

washed directly into lakes and rivers or other bodies of water by heavy rains; hence the presence of excreta on water sheds and consequent contamination of impounded waters is of considerable practical importance. In many instances, particularly in the case of shallow wells and the like, typhoid and other bacilli enter the water supply from privies, latrines, and similar devices via ground water. The distance over which such contamination can travel is a function of the rate of death of the bacteria and the rate of ground water flow. The latter is obviously dependent upon many factors such as the amount of rainfall, the local geological formations, and the like, and each instance of contamination, or possible contamination, must be considered individually. In general, fine soils and sand tend to impede their progress to a greater extent than coarse sand and gravel. The rock formations may be of considerable significance; sandstone, for example, filters out bacteria, while limestone tends to erode with the formation of direct communicating channels, and wells drilled into such formations may contain typhoid bacilli which entered the water many miles away and are, therefore, always to be regarded as dangerous. The rapid spread of suburban living, often beyond municipal water supply and sewage disposal systems, has resulted in a revival of the problems of ground water contamination[26] formerly confined to rural areas.

Contamination by excreta. Contamination of water by human excreta may take place, not only indirectly through the agency of soil as noted above, but also directly. Such direct contamination is, for the most part, a consequence of human population densities and urban organization and takes the form of dumping of sewage of one community into a body of water which serves as a water supply to another. Whether directly or indirectly contaminated, such waters contain not only the native bacterial flora supplemented by microorganisms from the soil, but also the flora of the human intestine. The contribution of the last consists of very large numbers of coliform bacilli, and much smaller numbers of *Cl. welchii*, the α-hemolytic fecal streptococci or enterococci (*Streptococcus fecalis*), together with whatever pathogens may be present. The pathogenic microorganisms are the pathogenic enteric bacteria such as the typhoid, paratyphoid (Salmonella); dysentery bacilli; where the disease is endemic epidemic, the cholera vibrio; certain of the animal parasites such as pathogenic amebae and Ascaris; and the so-called enteric viruses present in the bowel and excreted in the feces such as the polio and Coxsackie viruses and those of the ECHO group.

Under some conditions contamination by animal excreta assumes some importance. For example, surface water may contain coliform bacteria from domestic animals, and the presence of these does not carry the same implication as that of coliform bacteria of human origin. In other instances animal excreta may contain bacteria pathogenic for man, *viz.*, the contamination of water with leptospira from rat urine. Usually, however, contamination with animal excreta is not of great sanitary significance.

Factors Influencing the Kinds and Numbers of Bacteria. The numbers of bacteria that may be found in a given water are dependent primarily upon the type of water, whether it is a *surface water,* such as that found in streams, lakes, and shallow wells, or a *deep water* from deep driven wells. In the first instance opportunities for contamination are great and, as might be expected, many bacteria are present. The water from deep wells, on the other hand, has undergone effective filtration in order to reach the deeper strata in which it is obviously not subject to any extensive contamination; hence relatively few bacteria are found.

A variety of environmental factors influences the bacterial content of water; chief among these are the amount of organic matter present and the temperature. In general, the more nutriment there is present in the form of organic matter, the great the number of bacteria. Low temperatures are not conducive to

rapid growth and tend to keep the numbers of bacteria down, a factor that favors the survival of pathogens such as the typhoid bacillus which are unable to multiply in any case. Higher temperatures result in an increase in bacterial numbers in the presence of sufficient organic matter, but if the supply of nutriment is not great, after a preliminary increase during which the food supply is exhausted, the numbers fall below the initial level. Other environmental factors are not infrequently influential in determining the types of bacteria present in a water; thermophiles will, of course, predominate in hot springs and sulfur bacteria in sulfur springs, and the acidity of many natural waters results in a limited flora of acid-resistant bacteria.

Bacteria in ice. Although it is difficult if not impossible to sterilize a substance by exposure to low temperatures, many of the bacteria present are killed, only the resistant cells surviving. The great majority of bacteria in water are killed by freezing. Hence ice always contains but a fraction of the number in the water from which it was formed. Over 90 per cent both of the ordinary water bacteria and of typhoid bacilli die within a few hours, and a progressive decline in numbers then takes place, less than 1 per cent of typhoid bacilli surviving at the end of a week of freezing. Ice stored for six months is practically sterile. Outbreaks of typhoid fever have rarely been traced to the use of ice, although in a few instances the evidence of ice transmission seems quite conclusive. Danger of typhoid infection from the use of ice in drinking water is, in the absence of direct contamination of the ice during handling, always less than from the use of water from the same source as the ice.

The Bacteriological Analysis of Water.[1, 17] It will be apparent from the above discussion that the bacteria whose presence in water is a consequence of fecal pollution are not present in uncontaminated water and are sufficiently different from the native water bacteria that they may be readily distinguished. Of these bacteria the coliform bacilli are present in greatest numbers, while *Str. fecalis* and *Cl. welchii,* although constantly present, are usually not so numerous. It would appear, therefore, that any of these microorganisms could be used as an indicator of pollution. The coliform bacilli are used almost exclusively in this country, and both *Str. fecalis* and *Cl. welchii* have been used in Europe. The streptococci are, however, sometimes difficult to differentiate and die out more rapidly than coliform bacteria. *Cl. welchii* has the disadvantage that its spores remain viable over long periods in contrast to coliform bacilli which, although more hardy than the typhoid bacillus, die out in time; hence *Cl. welchii* does not allow the differentiation of recent and old pollution.

The bacteriological examination of water for the presence of coliform bacilli rests upon the fact that this microorganism ferments lactose. The standard procedure for the examination has been prepared jointly by the American Public Health Association and the American Water Works Association and is revised at frequent intervals. Briefly, it consists of three parts, (1) the presumptive test, (2) the confirmed test, and (3) the completed test. In the first, lactose broth is inoculated with decimal dilutions of the water sample, commonly 10 ml., 1 ml., and 0.1 ml. (expressed as dilutions these are, respectively, 0.1, 1, and 10). The volume of the smallest inoculum producing fermentation provides a crude approximation of the numbers of coliform bacilli present in the water. Of the selective enrichment mediums containing ox bile and/or brilliant green or ricinoleate or lauryl sulfate, only lauryl sulfate tryptose broth has been officially accepted for the presumptive test without confirmation, and then not for filtered or treated waters. A more accurate estimate may be obtained by inoculating five tubes with each dilution and calculating the most probable number of coliform bacilli on the basis of the number of tubes in which fermentation occurs.[21, 25] Tables for calculation of the most probable numbers (MPN) are given by Prescott, Winslow, and McCrady.[19] The

confirmed test consists of the inoculation of a specified selective medium such as Endo or eosin–methylene blue (EMB) plates, brilliant green lactose bile broth, crystal violet lactose broth, fuchsin lactose broth, or formate ricinoleate broth. The appearance of typical coli colonies on the plates or fermentation in the selective lactose broth constitutes a positive confirmed test. In the *completed test* one or more typical colonies are picked from an Endo or EMB plate inoculated either from the original lactose broth culture or from the secondary selective medium showing fermentation and are transferred to an agar slant and a lactose fermentation tube. After incubation the slant culture is smeared and stained and examined for the gram-negative non-spore-forming rods of coliform bacilli. If the culture is found to be morphologically coliform bacilli and the lactose is fermented, the completed test is positive.

Membrane filter procedure.[5] This method of enumerating bacteria in water, and in other fluids for special purposes such as quantitative blood culture, was introduced by Goetz in Germany in 1947 and subsequently was widely studied in this country. The microbial indicator is usually coliform bacilli, but enterococci may be enumerated also.[20]

It consists essentially of the filtration of the water sample by negative pressure through a cellulose disc which will retain the bacteria,* removal of the filter disc to an absorbent pad** containing a differential liquid medium such as Endo medium in the case of coliform bacilli, and incubation. The bacterial colonies grow upon the surface of the filter disc and may be counted.

Duplicate 100 to 500 ml. volumes of finished, *i.e.*, regarded as potable, water, single filtrations of each of 0.1, 1, and 10 ml. volumes of well water, and appropriately reduced amounts of polluted waters are examined. The volume to be filtered is not less than 20 ml., and when the sample is smaller than this it must be diluted to 30 ml. with sterile buffered water. The filter disc, sterilized in the autoclave prior to use, is removed after filtration and rolled, avoiding the entrapment of air bubbles, onto the absorbent pad containing nutrient medium in a sterile petri dish. The membrane is incubated for two hours and then transferred to a fresh pad saturated with the nutrient medium, incubated overnight, and the colonies counted.

The coliform counts so obtained are somewhat lower than those estimated by the MPN method. Whether the two methods do not measure precisely the same microorganisms, or whether the difference is a result of the mathematical bias[23] of the MPN method, which is reported to give estimates 20 to 25 per cent higher than the actual coliform density, is not yet clear.

This is the direct membrane filter test. When completed test procedures described above, *i.e.*, representative colonies, are examined further and shown to be non-spore-forming gram-negative bacilli which produce gas in lactose fermentation tubes, it becomes the verified membrane filter test.

The microbial indicator. It is self-evident that a satisfactory indicator of pollution must persist at least as long as, and preferably longer than, the pathogenic microorganisms that may accompany them. The microorganisms used, coliform bacilli, enterococci, and *Cl. welchii*, all persist longer than the pathogenic enteric bacilli, such as the typhoid bacillus, and the first may under some circumstances show a transitory multiplication. The survival of enteric viruses is similar to that of pathogenic bacteria, but at least some of them, such as Coxsackie viruses, may persist longer than coliform bacilli at low temperatures, 8 to 10° C.,[6] and these and other viruses such as poliovirus are more resistant to chlorination than coliform bacilli. In the case of treated waters, then, coliform counts may be reduced to "acceptable" levels even though some of the enteric viruses may persist. The matter

* Such as the Millipore type HA grid marked filters or their equivalent.

** Equivalent to Schleicher and Schuell No. 470 filter paper, approximately 48 mm. in diameter and capable of absorbing 1.8 to 2.2 ml. of nutrient medium.

of viral contamination of potable water has not yet been satisfactorily settled.

The casual and inclusive term coliform bacilli has been used advisedly above. The group is a heterogeneous one within the larger group of gram-negative enteric bacilli (Chap. 19) whose precise nomenclature continues to elude general agreement. Those which ferment lactose promptly are the microorganisms found in the foregoing tests for fecal pollution and include the coli and aerogenes types together with forms intermediate between them. These are placed in different genera, *e.g.*, *Escherichia coli* and *Aerobacter aerogenes* in this country, and in the genus Bacterium elsewhere, and it is becoming apparent that *A. aerogenes* as currently defined is contracting, the great majority of these forms becoming *A. cloacae* or *Cloaca cloacae*, depending upon the nomenclature followed.

Aside from matters of nomenclature, differentiation of these coliforms from one another is readily accomplished by biochemical reactions which are standardized for this purpose (Chap. 20). Whether such differentiation has any significance with respect to the sanitary quality of a water is another matter. It has been felt by some that the coli type is to be associated primarily with fecal contamination, while the aerogenes type may occur free in nature and that the latter has less sanitary significance. However, the aerogenes type also occurs in the bowel and is seldom found in the complete absence of possible fecal contamination of soils, and it is doubtful that the differentiation of the two types has significance in other than exceptional conditions.

As indicated above, the α-hemolytic streptococci of fecal origin, the enterococci, and *Cl. welchii* tend to be of uniquely fecal origin, and have been used as indicators of pollution. The enterococci have been of interest in this country in connection with the microbiological assay of swimming pool waters (see below), though here also the coliform bacilli have been used most often. In general, a lack of suitable culture mediums for the detection and enumeration of enterococci has been a practical difficulty, and a number of reasonably satisfactory culture mediums have been developed.[16] Such mediums are made selective by the inclusion of sodium azide, and differential with certain dyes such as ethyl violet or triphenyl tetrazolium chloride, and the membrane filter method may be applied.[20] Enterococci tend to persist longer than pathogenic enteric bacteria, but not as long as coliforms, and are usually found in considerably smaller numbers than coliform bacilli.

Plate counts. It is usually desirable to have an approximate measure of the total number of bacteria in drinking water, not because the sanitary quality of a water can be judged on this basis alone, but because such information frequently has ancillary value. The counts obtained by quantitative dilution and plating are, it must be remembered, those of the microorganisms that will grow on the medium used, other bacteria being quite inapparent by this method.

Two series of plates are poured, one in which the medium is nutrient gelatin and the other nutrient agar, or both may be nutrient agar. The gelatin plates, or one set of agar plates, are incubated at 20° C. and the agar plates at 37° C. In general the native water and soil bacteria grow best at 20° C.; in some cases they do not grow at all at 37° C.; and bacteria of animal origin grow most rapidly at body temperature. The relative numbers of microorganisms growing at the two temperatures are, then, at times suggestive of the origin of the bacteria found.

Chemical Analysis. The analysis for appropriate chemical compounds frequently is of value as an adjunct to bacteriological analysis in the determination of the sanitary quality of a water.[1, 17] Pollution by sewage, for example, adds complex compounds—protein, carbohydrate, and fat—to the water, and the amount and state of the decomposition products of these substances may serve as an index of the degree and

time of pollution. Ammonia, nitrites, nitrates, chlorides, and albuminoid nitrogen are usually determined. Of these, chloride and, to a certain extent, nitrate, are the most useful. Chemical analysis of water is frequently made in connection with hardness, turbidity, taste, smell, and similar features, which, while often of considerable industrial or esthetic significance, are of no sanitary importance.

The Assay of the Sanitary Quality of Water.[22] The means by which the sanitary quality of a water is judged may be summarized briefly:

(1) the bacteriological analysis, including both
  (a) the presence or absence of coliform bacteria and
  (b) the number and type of bacteria present
(2) the type of water, whether surface or deep
(3) the local conditions and
(4) chemical analysis.

Of these the presence and numbers of coliform bacteria are the most important, and it must be remembered that the relative abundance, rather than the presence, of these microorganisms is the essential feature of the test. The discovery of a single colon bacillus in 50 ml. of water, or even occasionally in 5 ml., affords no reasonable ground for suspicion of the water. The possibility of sporadic contamination with colon bacilli derived not from man but from domestic animals or birds must be kept in mind. Manured fields and pastures, filled with grazing cattle or sheep, are likely sources of colon bacilli and may give rise to mistaken inferences if the environmental examination of a water supply is neglected. Knowledge of such local conditions as well as the type of water is, then, essential to the interpretation of the bacteriological findings. Chemical analysis may be of considerable help in some instances, but in general finds its greatest utility in the study and control of gross pollution in which the decomposition of organic matter and presence of industrial wastes are a nuisance, rather than the assay of the purely sanitary quality of a water.

Drinking waters. The question of the significance of the bacteriological findings brings up the matter of standards to which a water suitable for drinking should conform. Now it will be clear that, although considerable numbers of colon bacilli in a water are always suggestive of fecal contamination by man or animals, a standard is necessarily a minimum which is inherently difficult to define under the circumstances. In this country the United States Public Health Service has prepared recommended standards[24] which are intended to represent a minimum. In the recommended procedure the frequency of sampling is dependent upon local conditions, the minimum varying from one per month for a population of 2500, to 500 per month for a population of five million. The sample is five 10 ml. portions, or five 100 ml. portions. The results of bacteriological examination by Standard Methods procedures should be as follows:

(1) Of all 10 ml. portions examined per month, not more than 10 per cent shall show the presence of coliform bacteria.
(2) Occasionally more than three of the five samples show coliforms; this must not occur in more than 5 per cent of samples when twenty or more samples are taken per month, or in more than one sample if less than twenty samples are taken per month.
(3) Should such a result (as in 2) be obtained from a single standard sample, daily testing must be carried out until at least two consecutive satisfactory samples have been found. Such daily samples are to be regarded as "special samples" and not included in the monthly totals.
(4) With regard to the 100 ml. samples:
  (a) Not more than 60 per cent shall show coliforms.
  (b) Occasionally all five portions constituting a single sample will show coliforms; this must not occur in more than 20 per cent of samples when five or more samples are examined per month, or in more than one if less than five samples are taken per month. If so, daily "special samples" must be taken as above.

The water shall be satisfactory as to taste, odor, and color and shall contain less than the following minimum quantities of chemical impurities: lead, 0.1 ppm; fluorine, 1.5 ppm; arsenic and selenium, 0.05 ppm; copper, 0.3 ppm; iron and manganese, 0.3 ppm; magnesium, 125 ppm; zinc, 15 ppm; chloride and sulfate, 250 ppm; total solids, 500 ppm; phenol, 0.001 ppm.

The British Ministry of Health sug-

gests standards[17] based on the presumptive coliform count as determined by acid and gas formation in MacConkey broth on the piped supply entering the distribution system. Waters are divided into classes on the following basis:

Class I—Water of class I and regarded as highly satisfactory contains less than 1 coliform per 100 ml.

Class II—Water regarded as satisfactory contains 1 to 2 coliforms per 100 ml.

Class III—Water regarded as suspicious contains 3 to 10 coliforms per 100 ml.

Class IV—Water regarded as unsatisfactory contains more than 10 coliforms per 100 ml.

While a presumptive test alone is specified, the occurrence of positive presumptive tests has been found to be very high in Britain. Waters of classes I and II conform closely to the American standards.

The World Health Organization has compiled standards in the sense of bringing together common practices and criteria of water examination, together with suggested procedures;[27, 28] in general these resemble closely those described above.

Swimming pools and bathing places.[15] The sanitary control of water in swimming pools and bathing beaches is similarly based on bacteriological examination. The American Public Health Association has recommended that not more than 15 per cent of samples of swimming pool water contain more than 200 bacteria per ml. or give positive confirmed tests for coliforms in any of five 10 ml. samples when the pool is in use. The standard is as high as that for drinking water, but pools are usually filled with water of drinking quality, and pollution is not only derived from bathers but is fresh and may be highly infective. The presence of acid-forming streptococci is also of considerable utility as a measure of oral and skin contamination, and usually corresponds closely to the total count. Such waters commonly contain residual chlorine which must be neutralized with thiosulfate when samples are collected for bacteriological examination. In natural outdoor bathing places the test for coliforms is the most important. The standards are necessarily much more lenient than those for indoor pools, and those adopted locally vary from an allowable 100 coliforms per 100 ml. in California and Indiana to 3000 coliforms per 100 ml. allowed by the New York City Health Department.

The Purification of Water Supplies. When, by bacteriological examination or otherwise, a water is known to be unsafe for consumption, the question arises as to ways and means of artificial purification. There are a number of useful methods of purifying water, differing according to the amount and character of the water to be treated, which may be summarized as follows:

(1) mechanical methods
  (a) storage
  (b) filtration
    1. slow sand filtration
    2. coagulation and rapid sand filtration
(2) chemical methods
  (a) large scale—hypochlorites and liquid chlorine
  (b) small scale—hypochlorite, ultraviolet light, ozone, etc.

Of these, storage is not generally regarded as a method of water purification, though the numbers of bacteria are usually greatly reduced in impounded waters because of the exhaustion of the food supply and the consequent death of bacteria and settling, not so much of bacteria alone as of suspended matter which carries down bacteria with it. The partial removal of suspended matter is frequently desirable, particularly with turbid waters, and may be carried out by allowing the water to remain in a settling basin for a time.

Slow sand filtration is one of the earliest and most effective methods of water purification and is in use in many European and some of the older American cities. These sand filters are constructed so that the water passes through 1 to 5 feet of sand supported upon graded layers of gravel. The rate of filtration must be accurately regulated and the efficiency of operation controlled by frequent bacterial test of the effluent. Such filters are highly effective. Bacteria are removed, not to any great extent by mechanical straining out, but through a

biological mechanism in which the activity of protozoa is an important feature. The passage of water through these filters is necessarily a relatively slow process and, in consequence, relatively large areas are required, which, for financial or other reasons, are no longer available in large American cities. Few of these filters have been constructed in recent years, and the use of rapid sand filters is becoming common.

The rapid sand filters, which may be used with turbid waters that would clog a slow sand filter, are frequently employed in conjunction with "coagulation," the addition of such substances as aluminum or ferric sulfate, which form flocculent precipitates (the hydroxides). The precipitate carries down most of the suspended matter and, of course, many bacteria, and is readily filtered out, yielding a clear effluent containing relatively few bacteria. Such flocculation also removes Coxsackie and bacterial viruses, to 85 to 95 per cent, under experimental conditions.[2] Such filters must be cleaned from time to time, a process that is seldom required with slow sand filters. They make possible, however, the treatment of a large quantity of water on a relatively small filtering area.

Destruction of pathogenic bacteria in water supplies is most often brought about by treatment with germicidal chemicals. Of these, hypochlorite (calcium hypochlorite or bleaching powder) was at one time used widely in the treatment of municipal water supplies, and it still has its uses, although it has been largely superseded in large-scale treatment by liquid chlorine, which is supplied in cylinders under pressure. Chlorine is added directly to the water through an automatic feeding device in accurately measured amounts which are determined by the character of the water. In general, the greater the amount of extraneous organic matter present, the greater must be the amount of chlorine added.

The amount of chlorine taken up is termed the chlorine demand, and the point at which the residual chlorine or available chlorine becomes proportional to the added chlorine is called the "breakpoint" in the chlorine demand curve. The introduction of breakpoint chlorination usually results in material improvement in the sanitary quality of a water. When liquid chlorine is added to ordinary surface water, clear and not highly contaminated, in the proportion of about 0.5 to 1 part of "available chlorine" per million gallons, the ordinary intestinal bacteria are destroyed, including such pathogenic forms as the typhoid bacillus. Certain of the enteric viruses, such as poliovirus, are more resistant to chlorination than are coliform bacilli, requiring a concentration of more than 0.2 ppm while a dose as low as 0.1 ppm may be sufficient to destroy the coliforms. The question of the persistence of enteric viruses in treated waters has been pointed up by the occurrence of water-borne epidemics of infectious hepatitis, and has not yet been fully clarified.[11, 12, 14]

The tastes and odors in chlorinated waters may be due to an overdose of chlorine caused by inadequate control methods or to the action of chlorine upon compounds present in the water, commonly as industrial wastes. Excess chlorine and tastes caused by chlorination can often be removed by dechlorination with SO_2 or treatment with $KMnO_4$ or activated carbon. The bactericidal activity of chlorine may be prolonged, particularly in waters containing considerable organic matter, by the simultaneous addition of liquid ammonia with the formation of chloramines.

The introduction of chlorination of municipal water supplies has, in practically every instance, resulted in marked reductions in the incidence of enteric infection, typhoid fever in particular, in this country, but the addition of chlorine or other bactericidal agents is not a cure-all. When a water is sufficiently heavily contaminated, bacteria embedded in particles of organic matter are not killed even in the presence of available chlorine. It is generally agreed

that a content of more than 50 coliform bacteria per ml. indicates pollution too great for successful chlorination.

Chlorine dioxide (ClO_2) also has been applied to the treatment of water. It has the advantages that it destroys algal tastes as well as the chlorophenol taste of certain chlorinated waters and oxidizes organic matter more rapidly than chlorine, thus allowing the maintenance of a chlorine residual in the distribution system. In water solution this substance is decomposed by light to chloric and perchloric acids and oxygen, and is more bactericidal for coliform bacteria than chlorine.

Frequently a combination of filtration and chlorination is desirable particularly with rapid sand filters. Not only does a preliminary filtration have esthetically desirable features in the case of turbid waters, but considerably less chlorine is required for treatment than would have been the case had not the greater part of the suspended material been removed.

The chemical treatment of water on a small scale does not always involve expense as a primary consideration, and hence other methods may be used. The treatment of swimming pool water by ultraviolet irradiation adds no tastes or odor to the water and, although considerably more expensive than chlorination, can be used because the scale of operation is small. Ozone, which is strongly bactericidal, is also relatively expensive and is not commonly used in this country but is used to an appreciable extent in Europe. Chlorination is, of course, always available. It is carried out by the addition of hypochlorite when the installation of liquid chlorine apparatus is not desirable, as, for example, in the treatment of a contaminated cistern.

At times the treatment of water becomes an individual matter, as in the case of an army in the field or when a public supply is known to be impure. In the first instance the water may be treated by the addition of hypochlorite in the form of bleaching powder or a solution of sodium hypochlorite or 2 drops of a 2 per cent tincture of iodine per quart. Hypochlorite tablets and solutions are available for this purpose, and iodine compounds, such as tetraglycine potassium triiodide, have also been made into tablets under trade names such as Globaline, Potable Aqua, and Individual Water Purification Tablets. The last are said to be effective against amebae cysts, cercariae, and certain of the viruses as well as against the common pathogenic enteric bacilli.[3] In the home, filters, such as the Berkefeld or Chamberland bougies, may be used, but filtration is slow, and care in operation and frequent cleaning are necessary. The simplest and best method of water treatment for the family or individual, however, is simple boiling. Boiling for 5 minutes is quite sufficient to destroy with certainty the typhoid bacillus and allied forms as well as the cholera vibrio. When water-borne disease is prevalent, or when a water supply is notoriously impure or exposed to chance of infection, boiling is the only wholly safe procedure.

SEWAGE

Sewage is best regarded as the used water supply of a community and as such is a dilute solution of fecal matter and other wastes. From the hygienic point of view it is an important vehicle in the transmission of enteric infection; hence the manner of its disposal is of considerable significance. The mechanisms of sewage disposal have as their objects: first, the ridding of a community of an ever-present volume of waste; and second, disposal in such a manner that it is not dangerous to other communities.

The complex organic compounds present in sewage undergo the same processes of decomposition that are involved in the breakdown of dead organic matter in nature and are a part of the so-called cycles of elements such as nitrogen, phosphorus, and the like. Any type of sewage treatment, then, is nothing more than a mechanism for bringing about or accelerating these transformations. The organic compounds are first broken down to amino acids, monosaccharides, and

the like, which are eventually oxidized completely to carbon dioxide and water in the case of carbon and hydrogen, and to nitrite and nitrate in the case of nitrogen. Bacteria are the active agents in this decomposition and oxidation, the mechanisms of which have been discussed earlier (Chap. 5). Although essentially simple in principle, sewage treatment is in practice a complex problem which cannot be considered at length here.[4, 18]

In general, however, sewage disposal falls into one of three categories: (a) dilution, (b) partial treatment, and (c) complete treatment. In the first instance the sewage is simply dumped into some body of water where it will not annoy its originators. Here the breakdown and oxidation of the constituents of the sewage occur in nature, and, if sufficient time elapses, no trace remains beyond an increase in nitrate which, in turn, serves as food material for phytoplankton. When this transformation takes place in a flowing stream, the phenomenon is known as the *self-purification of streams*. The essential element, however, is not the fact that the water is in movement but that sufficient time elapses for the breakdown and oxidations to proceed to completion. Soil polluted with sewage similarly "purifies itself."

With increasing population densities the disposal of sewage by dilution becomes unsatisfactory because a body of water is not infrequently a water supply to a neighboring community. Some form of treatment, then, becomes obligatory; in other words, the decomposition is made to take place in whole or in part in the various tanks and other devices making up a sewage treatment plant rather than allowing it to occur in natural bodies of water. In practice, treatment takes the form of preparatory processes followed by a period of anaerobic digestion, then a period of aerobic oxidation. Treatment may be complete even through nitrification, or the partially treated sewage may be disposed of by dilution.

When the disposal of fecal material is an individual or family problem the mechanics involved are somewhat different, but the processes of decomposition and oxidation are essentially the same whether a privy, cesspool, or septic tank is used.

It will be noted that, although sewage treatment is basically a bacteriological process, the pathogenic bacteria are not involved; their presence is not taken into consideration, nor is there any effort to destroy them except in the rare instances in which the effluent from a complete treatment plant is chlorinated. In one sense, then, sewage treatment is not directed toward the control of water-borne disease. The fact that it is treated, however, is of considerable significance in this connection. When sewage is disposed of by dilution, the typhoid bacillus and related microorganisms, together with enteric viruses, do not multiply; rather, a decline in numbers sets in immediately, and these bacteria do not survive as long in water as in soil, probably not more than a few days or a week and they tend to disappear during sewage treatment, although there is no assurance that pathogens are absent in the effluent from treatment plants.[9, 10, 13] Clearly then, fresh pollution is more dangerous than past pollution, and, in a sense, treatment obviates the possibility of such fresh pollution. The economic and esthetic aspects of sewage treatment are, of course, another matter.

REFERENCES

1. American Public Health Association: Standard Methods for the Examination of Water and Wastewater. American Public Health Association, New York. 11th ed., 1960.
2. Chang, S. L., et al.: Removal of Coxsackie and bacterial viruses in water by flocculation. I. Removal of Coxsackie and bacterial viruses in water of known chemical content by flocculation with aluminum sulfate or ferric chloride under various testing conditions. Amer. J. Pub. Health, 1958, **48**:51–61.
3. Clark, R. N.: The purification of water on a small scale. Bull. W.H.O., 1956, **14**:820–826.
4. Eckenfelder, W. W., Jr., and O'Connor, D. J.: Biological Waste Treatment. Pergamon Press, New York. 1961.
5. Ehrlich, R.: Application of membrane filters Adv. Appl. Microbiol., 1960, **2**:95–112.

6. Gilcreas, F. W., and Kelly, S. M.: Relation of coliform-organism test to enteric-virus pollution. J. Amer. Water Works Assn., 1955, **47:** 683–694.
7. Henrici, A. T.: The distribution of bacteria in lakes. In Problems of Lake Biology, Amer. Assn. Advancement Sci., Publication No. 10, 1939.
8. Heukelekian, H.: Microbiology of water and sewage. Ann. Rev. Microbiol., 1953, **7:**461–472.
9. Kabler, P.: Removal of pathogenic microorganisms by sewage treatment processes. Sewage Indust. Wastes, 1959, **31:**1373–1382.
10. Kelly, S. M.: Enteric virus isolations from sewage. Acta Med. Scand., 1957, **159:**63–70.
11. Kelly, S. M., and Sanderson, W. W.: Chlorination of poliovirus. Science, 1957, **126:**560–561.
12. Kelly, S. M., and Sanderson, W. W.: The effect of chlorine in water on enteric viruses. Amer. J. Pub. Health, 1958, **48:**1323–1334.
13. Kelly, S. M., and Sanderson, W. W.: The effect of sewage treatment on viruses. Sewage Indust. Wastes, 1959, **31:**683–689.
14. Kelly, S. M., and Sanderson, W. W.: The effect of chlorine in water on enteric viruses. II. The effect of combined chlorine on poliomyelitis and Coxsackie viruses. Amer. J. Pub. Health, 1960, **50:**14–20.
15. Lehr, E. L., and Johnson, C. C., Jr.: Water quality of swimming places. A review. Pub. Health Repts., 1954, **69:**742–748.
16. Litsky, W., Mallman, W. L., and Fifield, C. W.: A new medium for the detection of enterococci in water. Amer. J. Pub. Health, 1953, **43:** 873–879.
17. Ministry of Health (British): The Bacteriological Examination of Water Supplies. H. M. Stationary Office, London. 3rd ed., 1956.
18. Porges, N.: Newer aspects of waste treatment. Adv. Appl. Microbiol., 1960, **2:**1–30.
19. Prescott, S. C., Winslow, C. E. A., and McCrady, H. Water Bacteriology. John Wiley and Sons, New York. 6th ed., 1946.
20. Slanetz, L. W., Bent, D. F., and Bartley, C. H.: Use of the membrane filter technique to enumerate enterococci in water. Pub. Health Repts., 1955, **70:**67–72.
21. Stevens, W. L.: Dilution series: A statistical test of technique. J. Roy. Statist. Soc., Ser. B, 1958, **20:**205–214.
22. Streeter, W. H.: Laboratory control of water supplies. Pub. Health Repts. Suppl. No. 201. 1948.
23. Thomas, H. A., Jr., Woodward, R. L., and Kabler, P. W.: Use of molecular filter membranes for water potability control. J. Amer. Water Works Assn., 1956, **48:**1391–1402.
24. United States Public Health Service: Manual of Recommended Water Sanitation Practice. United States Public Health Service, Washington. Public Health Bulletin No. 296. 1946.
25. Woodward, R. L.: How probable is the most probable number? J. Amer. Water Works Assn., 1957, **49:**1060–1068.
26. Woodward, F. L., Kilpatrick, F. J., and Johnson, P. B.: Experiences with ground water contamination in unsewered areas in Minnesota. Amer. J. Pub. Health, 1961, **51:**1130–1136.
27. World Health Organization: International Standards for Drinking Water. World Health Organization, Palais des Nations, Geneva. 1958.
28. World Health Organization: European Standards for Drinking Water. World Health Organization, Palais des Nations, Geneva. 1961.
29. Zobell, C. E.: Marine Microbiology. Chronica Botanica, Waltham, Massachusetts. 1946.

Chapter 12

THE MICROBIOLOGY OF MILK AND FOOD

Other than the ramifications of a common water supply, the most common vectors of disease of microbial etiology are the wide variety of foods consumed by man. Like water, these constitute a link between the susceptible individual and the source of infection.

MILK[8, 9, 11, 17]

As a vector of infectious disease, milk differs from water in that it is an excellent medium for the growth of many pathogenic bacteria, and from other foods in that it is the only food of animal origin that is consumed in large part in the raw state. Since large quantities of milk are consumed—it is estimated that about 16 per cent of the average dietary in the United States consists of milk and milk products—the importance of this substance in the transmission of disease is evident. The diseases transmitted by milk are: first, diseases of cattle transmissible to man and including bovine tuberculosis, undulant fever, foot-and-mouth disease, and streptococcal infections from infected udders; and, second, diseases of man in which the milk serves as the link between man and man, such as typhoid fever, diphtheria, and, rarely, certain other diseases such as poliomyelitis.

Sources of Bacteria in Milk. Unlike water, milk has no native bacterial flora, and it is probable that milk as secreted into the udder of a healthy cow is sterile. The milk in the udder is, however, rarely if ever bacteriologically sterile, for the microorganisms invade the udder via the milk ducts of the teats and the first portion of the milk drawn (foremilk) always contains more bacteria than the last (strippings). There is, furthermore, no bacterial flora that is characteristic of milk; the presence of microorganisms is always a consequence of contamination, and the types of bacteria found are determined by the source of contamination. From this point of view the bacteria of milk fall into two groups: first, those which are present in the tissues of an infected cow and find their way into the udder; and, second, those which enter the milk, usually after it is drawn, from sources external to the animal.

Bacteria from Infected Cattle. Of these microorganisms perhaps the most important is the tubercle bacillus. These

bacteria get into the milk directly as a consequence of tuberculosis of the udder, which occurs in 1 to 2 per cent of infected cows, and indirectly by contamination with cow manure when the infectious sputum is swallowed and discharged with the feces. In either case the milk is infective for man, the bovine variety of the tubercle bacillus giving rise, as a rule, to bone and joint rather than pulmonary tuberculosis. Milk may, of course, be infected with human tubercle bacilli from infected persons. A high proportion of the former type of infection occurs when the transmission of this disease is allowed to occur. The disease may be controlled at its source, *i.e.*, by the elimination of infected cattle from dairy herds, a practice which is practically universal in the United States.

A bovine variety of *Brucella melitensis,* the causative agent of undulant fever, *Br. abortus,* infects cattle, producing the disease contagious abortion. This microorganism is excreted in the milk and infects man, producing a mild type of undulant fever. The caprine variety of this bacterium which is present in the milk of infected goats produces a much more severe disease in man. Undulant fever, like tuberculosis, may be controlled by control of the disease in the animal reservoir of infection.

The virus of foot-and-mouth disease, a virus disease of cattle, is excreted in the milk and is thus transmitted to man. The disease in man is mild, however, and not of great public health importance. More important are the streptococcus infections of the udder, designated garget or mastitis. The Hotis test is commonly used for the detection of mastitis; it consists of incubating fresh milk in the presence of 0.025 per cent bromcresol purple for 24 hours at 37° C. A positive reaction, the formation of yellow flakes on the side of the test tube, is dependent upon the presence of the streptococci and of agglutinins in the milk; the growing bacteria are clumped by the antibody and the acid reaction results from the fermentation of lactose. The streptococci of bovine mastitis, *Str. agalactiae,* are rarely found to be a cause of human disease, but milk-borne epidemics of streptococcal infection may be associated with acute udder inflammation in the dairy herd, and the massive and continuous infection occurring in some of the outbreaks of human streptococcal infection indicates that the udders of the cattle were infected. It has been suggested that *Str. pyogenes* may proliferate, with or without symptoms, in the udder, and epidemics of human streptococcal infection may occur, though it is probable that in most instances milk-borne infection is a consequence of direct human contamination. The udder may be infected occasionally with diphtheria bacilli, which produce small external ulcers, but this is an uncommon occurrence.

Bacteria from External Sources. When milk is collected under ordinary conditions, the udder bacteria form but an insignificant fraction of the total number of microorganisms in the milk. The skin of the cow, the hands of the milker, the vessels used for collection, and the dust of the cow barn all contribute their quota to the number of bacteria found immediately after milking. If milk is obtained with aseptic precautions, it contains only a few hundred (200 to 400) bacteria per ml.; collected with somewhat less care, it may contain a few thousand (2000 to 6000); with careless manipulation, even freshly drawn milk may be highly contaminated (30,000 to 100,000 per ml.). If milk is kept at 0° C. (32° F.), it shows a decrease in the bacterial content during the first few hours, but at higher temperatures the rate of multiplication is high and, when richly seeded at the outset, enormous numbers of bacteria result.

The nonpathogenic bacteria that are present in milk are often differentiated on a physiological basis into the following groups:

(1) the acid-forming bacteria
(2) the alkali-forming bacteria
(3) the proteolytic bacteria
(4) the inert bacteria.

The first group includes the fermentative bacteria, and the most common type

of fermentation is the lactic acid fermentation, the process by which milk usually sours under natural conditions. A variety of bacteria may be responsible, among them *Staphylococcus aureus, Str. pyogenes,* and *Escherichia coli.* A few species, however, are commonly active in the natural souring of milk, and these may be divided into two groups. One of these is comprised of the capsulated gas-forming bacilli of the *Bacterium (lactis) aerogenes* type, which are closely related to *E. coli,* differing principally in their possession of capsules, lack of motility, and ability to produce gas from potato starch. The second type, a streptococcus, *Streptococcus lacticus* (or *lactis*), is abundant in naturally soured milk, particularly when the acidity is high.

Lactic acid milks, regarded by some as having therapeutic value in the treatment of certain intestinal disorders, have been prepared by the inoculation of Lactobacillus species such as *L. acidophilus* and *L. bulgaricus.* The lactobacilli are commonly found in the fermentation of ensilage and are present in small numbers in the human mouth and intestinal tract and are not ordinarily responsible for the natural souring of milk.

Although lactic acid is commonly the predominating acid in the fermentation of milk, the formation of butyric acid is observed occasionally. This is generally a consequence of the presence of the anaerobic butyric acid bacteria but may be brought about by *Bacillus subtilis* and related spore-forming aerobic bacilli.

The spontaneous alcoholic fermentation of milk is less usual under natural conditions than either the lactic or the butyric, and the preparation of certain alcoholic beverages is dependent upon the artificial production of this form of milk fermentation. Koumiss, a drink prepared by Tartars by the alcoholic fermentation of mare's milk, and kefir, an effervescent sour milk prepared by inhabitants of the Caucasus from the milk of cows, goats, and sheep, are prepared by the inoculation of fresh milk with old koumiss in the first instance and with "kefir granules" in the second. The bacteriology of the koumiss fermentation is not well known, but in the case of kefir both a bacterium and a yeast appear to be involved. Some species of yeast are able to effect the alcoholic fermentation of milk in pure culture.

The alkali-forming bacteria are those organisms which do not ferment lactose but, presumably, act upon the nitrogenous substances present with the liberation of ammonia. When typhoid and paratyphoid bacilli, for example, are cultivated in litmus milk no effect is apparent beyond a slowly increasing alkalinity. Certain other bacteria, such as some of the aerobic spore-forming types, also produce lipase and decompose the fats present, converting the milk to a yellow, transparent fluid.

The proteolytic bacteria also produce an alkaline reaction and, in addition, hydrolysis of the milk proteins. Two enzymes, or groups of enzymes, are responsible; one, a rennin-like enzyme, precipitates the protein with the formation of a soft curd, and a second, casease, brings about hydrolysis of the protein which, when complete, results in the conversion of the milk to a clear fluid, a process sometimes termed peptonization. Peptonization, however, does not follow precipitation when the microorganism does not possess casease. The bacteria producing these changes include spore-forming aerobes such as *B. subtilis,* certain strains of staphylococci, *Proteus vulgaris,* and others. Proteolysis may occur in heated milk in which the non-spore-forming lactic fermenters have been destroyed, leaving the more resistant spore-forming proteolytic forms.

The "diseases" of milk. A series of unusual or abnormal changes, called "diseases" of milk, sometimes occur. "Blue milk," "red milk," and "yellow milk" are caused by the presence of various chromogenic bacteria. "Bitter milk," characterized by a bitterness that sometimes develops after a short interval, is likewise due to the products of certain microorganisms. Milk sometimes suffers from a ropy or slimy fermentation which, under most circumstances.

is regarded as undesirable, although such a fermentation is intentionally produced in the manufacture of Edam cheese in Holland through the action of a particular species of streptococcus.

The inert bacteria are those which produce no visible change in milk. These include certain non-pigment-forming bacteria from water and other sources, and, in addition, most of the pathogenic bacteria found in milk. Such dangerous contamination, then, is inapparent without bacteriological examination.

The Pathogenic Bacteria from External Sources.[16] The multiplicity of sources of contamination results in the heterogeneous bacterial flora that may be found in milk. It is important from the hygienic view that, in addition to microorganisms of soil and water, the bacteria carried by man have relatively ready access to milk. The contamination with the bacteria of human disease is a constant possibility, not only when the milk is drawn but throughout its handling until it reaches the ultimate consumer. Contamination may be direct, as in the case of streptococci or diphtheria bacilli from the throat and typhoid bacilli from the hands of infected individuals, or it may be indirect, as when the water used to wash the milk cans is contaminated with typhoid bacilli. In any case, the microorganisms do not simply survive, as in water, but actively multiply and may be present in huge numbers in pooled milk of which but a single part was originally contaminated.

Clearly, then, the most important factors which govern the number of bacteria which may be present in milk are, first, the kind and degree of initial contamination, and second, the temperature at which the milk is kept. The production of hygienically satisfactory milk, therefore, involves cleanliness in the first instance, and immediate cooling and storage at a low temperature in the second.

The Bactericidal Property of Fresh Milk. As indicated above, the number of bacteria present in freshly drawn milk frequently shows an initial decrease. Its extent may be exaggerated by plate counts; the various antibodies are present in the milk, not only the bactericidal substances but the agglutinins as well, which, by clumping the bacteria, may bring about decreased plate counts. That freshly drawn milk is both bactericidal and bacteriostatic, although to a mild degree, is, however, definitely established. This activity is thermolabile, being destroyed in 15 minutes at 75° C. and in 2 minutes at 80° to 90° C., and disappears a few hours after the milk has been drawn.

The Determination of the Quality of Milk. By far the best index of the quality of milk is the number of bacteria it contains. The bacteria present are, in all cases, a result of contamination, and hence these microorganisms are reliable

Milk-Borne Epidemics of Disease*

DISEASE	OUTBREAKS		CASES	
	Number	Per Cent of Total	Number	Per Cent of Total
Typhoid and paratyphoid fever	76	45.2	1,209	12.1
Septic sore throat and scarlet fever	57	34.0	6,812	68.2
"Gastroenteritis"	24	14.3	1,423	14.2
Bacillary dysentery	5	3.0	411	4.1
Diphtheria	5	3.0	123	1.2
Poliomyelitis	1	0.6	11	0.1
Totals	168	100.0	9,989	100.0

* In New York State exclusive of New York City as reported by Dublin, Rogers, Perkins, and Graves, [7]

indicators of cleanliness and care and are generally used for this purpose.

Plate counts. The total bacterial count of a milk is a reflection of its hygienic quality, and the plate count has been, and still is, widely used in the bacteriological grading of milk. Standardization of the mediums and of the technique is necessary for comparable results. A standard procedure has been developed under the auspices of the American Public Health Association.[2]

Standards of milk quality in terms of plate counts are in general use but vary from one locality to another. In some instances more than one grade of milk is allowable; for example, a "Grade A" milk may contain not more than 30,000 bacteria per ml. as delivered, and a "Grade B" milk not more than 100,000. There is a steady movement toward more strict requirements, and the maximum number of bacteria allowed is continually reduced. More than one grade of milk is undesirable in that the distribution of inferior milk is allowed, and, when possible, a single standard is preferable. In Chicago, for instance, there is but one grade of milk, and it must not contain more than 10,000 bacteria per ml. as delivered to the consumer.

The membrane filter technique used for the bacteriological examination of water (Chap. 11) is not useful for the routine examination of milk. It is not possible to pass significant quantities of milk through the filter disc without clogging and if this method is applied the bacteria must first be removed by centrifugation and filtered after resuspension in buffer or saline. It may be applied to equipment rinse solutions, etc.

Microscopic counts. Besides the ordinary macroscopic colony count obtained by plating, a microscopic method devised by Breed is of value for many purposes. In the Breed method milk is taken in a capillary pipette discharging 0.01 ml. and is dried over an area of 1 sq. cm. on a glass slide. After washing out the fat with xylol and fixing with alcohol, the film is stained with methylene blue. The number of bacteria per square centimeter is estimated by counting a measured area. The ratio used in comparing the microscopic count with the standard plate count is 4 : 1, although it is recognized as inaccurate and variable. This direct microscopic count does not involve incubation and has proved of special value in judging the quality of fresh milk as delivered to milk-receiving stations.

Methylene blue reduction.[13] If a small amount of methylene blue is added to milk and the mixture incubated, the dye will, in time, be reduced to the colorless leuco base. Although freshly drawn milk has some small power to reduce this and other dyes, the reduction is, for all practical purposes, a consequence of the metabolic activities of contained bacteria. There is, then, a direct relation between the time required for reduction and the number of bacteria present, and the measurement of this reduction time provides a useful approximation of the quality of a milk. The method is best adapted to raw milk and has the great advantage that no special apparatus or training of the operator is required. Although milk cannot be graded with any degree of precision in this way, it is generally agreed that a milk decolorizing in less than 2 hours is poor in quality, while that which does not decolorize in 8 hours is excellent. Resazurin has been used to some extent in the place of methylene blue.

Coliform count. The coliform index of water pollution is not generally applicable to milk. Water contamination of sanitary significance is fecal, but this is not the case with milk; coliform bacilli, for example, would not be associated with the presence of the diphtheria bacillus in milk. Further, since the great majority of market milks contain cow feces, the estimates varying from 60 to 100 per cent, coliform bacilli in milk are probably most often of bovine origin. Although the coli count has been used widely in the past, it is not worth while except under special circumstances.

The isolation of pathogenic bacteria. In contrast to water, the isolation of pathogenic bacteria from milk is not only a practical but often a desirable pro-

cedure. The presence of tubercle bacilli, for example, can be conclusively shown only through their isolation, in this instance usually by guinea pig inoculation. Other pathogens, such as hemolytic streptococci and Brucella, may be isolated by culture. The isolation of these bacteria is not a routine procedure but may be carried out when an outbreak of disease is suspected of being milk-borne.

Cell count and sediment test. The number of leucocytes present in a sample of milk and the amount of contained dirt that can be strained out through a standard cotton disc are frequently of value in the assay of milk quality. Excessive numbers of leucocytes are present in the milk from infected udders, and when their presence is noted in Breed smears, mastitis is suggested. The amount of sediment that a sample of milk contains is an index of the extent to which it has been contaminated and is often, though not necessarily always, correlated with the bacterial count.

The Hygienic Control of Milk. The sanitary quality of milk may be controlled in one or both of two ways: in the first instance, by preventing to a considerable degree the contamination of milk and the multiplication of contained bacteria; and, in the second, by destruction of bacteria, the pathogenic forms in particular, already present in the milk.

Inspection. The periodic inspection of dairy farms is carried out by the local board of health in many places, and, although it does not insure the absence of pathogenic bacteria from milk, it is effective in increasing cleanliness and reducing the numbers of bacteria. A score card is frequently used, and a given dairy farm is rated according to a system of points.

Certified milk. One of the earliest attempts to avoid the dangers of milk-borne infection was the elaboration of methods designed to safeguard milk at every step in its production, collection, and distribution. To this end "Medical Milk Commissions" were established in a number of localities in the United States, usually under the auspices of the local medical society. Milk conforming to certain standards is certified by such a commission to be of high quality. The regulations, which are generally excellent, deal with such matters as the cleanliness of barnyard and dairy, the purity of the farm water supply, the proper sterilization of utensils, and the health of the cows and of the milkers. The bacterial content is limited to 10,000 per ml., and the milk must be delivered within 36 hours.

Certified milk is undoubtedly safer to use than milk collected and transported without suitable supervision, and the work of the milk commissions did much to improve dairy conditions in many parts of the country. At the same time, raw milk, certified or not, can never be regarded as protected against all chances of contamination; the difficulty—not to say impossibility—of making sure that no typhoid carriers and no persons suffering from a mild case of diphtheria or scarlet fever are ever employed in a dairy is self-evident. Outbreaks of diphtheria, paratyphoid fever, and other diseases have, in fact, been traced to certified milk. For this reason, as well as because of the relatively high cost of production, the use of certified milk remains limited.

Pasteurization. The process of destroying pathogenic bacteria in milk is by far the most satisfactory method of controlling milk-borne infection. The use of a temperature high enough to kill most microorganisms but not so high as to produce radical alterations in the substance heated was first applied by Pasteur to preserving wines without destroying their original flavor or bouquet. Although still widely used in connection with bottled beer and wines, the process of pasteurization is now used chiefly for the treatment of milk.

The temperature to which milk is raised and the time for which it is held there are dictated by the heat resistance of the bacterium to be killed. From the beginning attention has been directed primarily toward the tubercle bacillus, and it has been found that this bacterium is killed by exposure to a temperature of 140° F. for 20 minutes. In practice, then, a temperature of 142° to 145° F. for a period of 30 minutes provides an ade-

quate margin of safety, and these are the requirements that are usually specified. The technical aspects of such treatment of milk on a large scale are of prime importance, taking the form of prevention of foaming, proper design of valves to prevent "cool pockets" and dead ends, and the like. It might be supposed that a higher temperature or longer holding time resulting in a greater margin of safety would be desirable; this is not the case, for if either the time or the temperature is increased alterations take place in the physical state of the milk in which the fat is dispersed into smaller globules and will not rise to the top on standing. Such a disturbance of the "cream line" is of esthetic rather than sanitary significance.

Pasteurization may also be accomplished by the application of higher temperatures for shorter times, 160° to 161° F. for 15 seconds, in the so-called flash or HTST (high temperature short time) process. Unless the milk is preheated and the process precisely controlled, it may not be quite as effective as the holding process, and it has been reported that Brucella will survive HTST without preheating. There is also some tendency for the process to affect the physical qualities of the milk.

The plate count of milk is tremendously reduced by pasteurization, for not only are the pathogenic bacteria, such as the tubercle bacillus, *Br. abortus,* streptococci, and the like, destroyed, but the majority of other bacteria present as vegetative cells are killed also. There appears to be some question of the efficacy of pasteurization, particularly the HTST process, in the destruction of Q fever rickettsiae for, while this disease does not seem to be milk-borne, rickettsiae introduced experimentally are not invariably destroyed by either pasteurization process.

The phosphatase test is based upon the presence of the heat-sensitive enzyme phosphatase in milk. Since 96 per cent of the enzyme is destroyed by heating to 143° F. for 30 minutes, the amount remaining in a pasteurized milk may be used as an indicator of the pasteurization as carried out. The enzyme liberates phenol from phosphoric-phenyl esters, and, as originally proposed, the test consisted of the addition of disodium phenol phosphate and Folin's reagent, incubating 18 to 24 hours, and reading the blue color developed.

Not all bacteria are killed by pasteurization. Besides the resistant spore-formers, aerobic and anaerobic, certain streptococci are able to survive the pasteurizing temperature. These are the lactic acid–formers rather than the pathogenic forms, and pasteurized milk sours in the ordinary way on standing, though a longer period is required—*i.e.,* its keeping qualities are improved. If higher temperatures are used (180° F.), the lactic acid bacteria are killed and proteolysis occurs as the milk spoils. Thermophilic bacteria are frequently present in pasteurized milk in great numbers, for the temperature of pasteurization is an incubation temperature for these microorganisms. The slight acid metallic taste occasionally noticeable in pasteurized milk is often attributable to their activity.

The presence of thermophilic and other thermoduric microorganisms in milk is taken as indicative of faulty farm sanitation, provided that the equipment of the processing plant is not contaminated with these forms as is sometimes the case. On the other hand, the presence of psychrophilic bacteria in pasteurized milk, such as Pseudomonas, Alcaligenes, and Achromobacter, not only detracts somewhat from the keeping qualities of the milk, but is almost always an indication of poor plant sanitation practices, *i.e.,* represents post-pasteurization contamination.

The process of pasteurization, while effective in destroying pathogenic bacteria present in milk and, incidentally, increasing its keeping qualities, is not to be regarded as an excuse for the marketing of dirty and highly contaminated milk. There is some evidence, for example, that the growth of enormous numbers of bacteria, even though nonpathogens in the usual sense, is associated with summer diarrhea of infants. In most cases, therefore, sanitary regu-

lations specify not only the allowable number of bacteria in pasteurized milk as delivered but also an upper limit for raw milk which is to be pasteurized.

The Regulation of Milk Quality. The application of appropriate methods of rendering and keeping milk satisfactory from the hygienic point of view is, essentially, a social and legal problem rather than a scientific one. To this end appropriate ordinances are more and more generally incorporated into the legal structure of the community, and these, when adequately enforced, produce a marked reduction in milk-borne disease. About half the cities of over 1000 population grade milk and permit the sale of one grade of raw milk and one grade of pasteurized milk; of the total volume of market milk about 74 per cent is pasteurized, 99.4 per cent is from tuberculin-tested herds, and 35 per cent from abortion-tested herds. In general, sanitary regulations are somewhat more strict and more rigorously enforced in the large cities. In Chicago, for example, all milk is pasteurized, including certified milk, and this regulation is rigidly enforced.

Such practice has resulted in marked reductions in the number of bacteria in market milk. In 1901 the bacterial content of market milk in New York City varied from 300,000 in the coldest weather to 5,000,000 during the summer months; in Chicago (1904) the counts ranged from 10,000 to 74,000,000, and in Boston (1892) averaged 4,500,000. The incidence of milk-borne disease has correspondingly decreased; for example, in 1907–1915 there were in Massachusetts 2215 cases of typhoid fever which were traced to milk, but in 1919–23 only 297.

Milk products. Various foods made from milk, such as ice cream, butter, cheese, and the like, are potential vectors of disease when made from milk contaminated with pathogenic bacteria. The bacteria tend to die out upon storage although it has been found that the typhoid bacillus will survive for 3 months or more in butter, and tubercle bacilli have been found in butter and certain quick-ripening varieties of cheese. Ice cream may serve as a vector for typhoid fever, streptococcal infection, etc. Pasteurization of the mix is customary and generally at higher temperatures than those used for milk. Human infections with foot-and-mouth disease have been traced to contaminated butter and cheese, but the public health significance of these findings is problematical.

FOOD POISONING AND FOOD-BORNE INFECTION[4, 5, 18]

The diseases transmitted by milk and milk products may be disseminated by a variety of other foods; in addition to these, however, other types of illness may result from the ingestion of contaminated foods which make up that group of affections designated as food poisoning. The kinds of illness that may result from the ingestion of food may be summarized briefly:

(1) individual idiosyncrasies
(2) toxemia from foods, such as
  (a) foods naturally poisonous
  (b) foods into which poisons have been accidentally introduced
  (c) foods containing poisons of bacterial origin formed by
    1. *Clostridium botulinum* and
    2. staphylococci
(3) food-borne infection, including both
  (a) bacterial infections, such as
    1. typhoid fever, dysentery, cholera, *et al.*
    2. Salmonella infection
  (b) parasitic infections.

In some instances food serves simply as a vector in the transmission of diseases such as the parasitic infections and the enteric diseases. In the remainder, however, the clinical manifestations are those associated with food poisoning proper—vomiting, diarrhea, enteritis, and a greater or lesser degree of prostration. Although a number of types of food

poisoning given above are not bacterial in origin, their clinical character may simulate bacterial food poisoning, and these must be considered in any attempt to ascertain the etiology of a given outbreak. Hypersensitivity to a given food substance, for example, is frequently manifested as vomiting, and an outbreak confined to a family may be the result of familial tendency; similarly, the gastrointestinal disturbances following the ingestion of naturally poisonous foods such as toadstools, or foods contaminated with poisons such as arsenic or cyanide, are often indistinguishable from those induced by some poisons of bacterial origin.

Food Poisons of Bacterial Origin. Poisoning with food containing toxic substances of bacterial origin is very common, probably more so than is generally recognized. The term "ptomaine poisoning" is a misnomer that is both misleading and inaccurate. The organic bases such as putrescine, cadaverine, methylamine, and the like, called ptomaines, which result from the bacterial decomposition of protein, are not toxic when given by mouth. Neither are other decomposition products toxic *per os.* While a partially decomposed food may be esthetically unattractive, the innocuous nature of the products of decomposition is obvious when one considers the advanced state of decomposition reached by some cheeses. Toxicity is, on the contrary, attributable to the presence of substances synthesized by the bacteria whose presence may or may not be associated with obvious evidence of decomposition of the food substance.

Botulism. Of the toxic substances formed by bacteria in food the most powerful is the toxin of *Cl. botulinum.* In the United States canned foods provide the anaerobic conditions necessary for the germination of botulinus spores which have survived processing, while in Europe meat products, particularly the larger sausages, have been more frequently involved. The microorganism rarely infects man under natural conditions, and the disease is an intoxication resulting from the ingestion of preformed toxin present in the food substance. The disease is not of great public health importance—about 2000 cases have been reported in all—and the outbreaks are generally limited in scope, often being confined to a family circle of a few individuals. The case fatality is high, perhaps 60 to 80 per cent, and when outbreaks do occur they attract considerable attention. This type of food poisoning differs from the others in that the symptoms are not necessarily gastrointestinal; the toxin acts upon the peripheral nerves and the symptoms are those resulting from such damage. The use of botulinum antitoxin is discussed elsewhere.

Since both anaerobic conditions and a period of incubation are required for the growth of *Cl. botulinum* and the formation of toxin, fresh foods are not involved in this type of food poisoning. The toxin is destroyed by heat, hence freshly cooked foods do not contain it. Canned foods and certain meat products which are consumed either cold or after simply warming may contain botulinum toxin if the spores of this bacterium were originally present and not destroyed. Commercial processing as now employed in this country is adequate to destroy botulinum spores, and at the present time outbreaks of botulism are for the most part attributable to the consumption of home-canned, insufficiently heated foods. The presence of toxin is not necessarily associated with obvious signs of spoilage, though it is probable that toxin-containing foods are never entirely normal in appearance, odor, and the like.

Staphylococcus food poisoning. The marked gastrointestinal disturbances characteristic of food poisoning are often associated with the consumption of foods containing starch thickening, such as eclairs, cream puffs, and certain types of cake fillings, salad dressings and the like. Upon bacteriological examination, the incriminated food generally is found to contain enormous numbers of bacteria, sometimes staphylococci, other times bacteria of the colon-aerogenes group, streptococci, Proteus, and similar microorganisms.

The causal relation of staphylococci—

generally, though not necessarily, hemolytic strains of the *Staph. aureus* group—to food poisoning was suggested in 1914, and in 1930 this relationship was definitely established. These bacteria produce a soluble toxic substance, or enterotoxin, which gives rise to typical food poisoning symptoms in man and rhesus monkeys upon feeding and in kittens upon injection. There is considerable doubt as to the specificity of the kitten test, however, for it has been shown that the staphylolysins will cause vomiting on injection into these animals. The incubation period in man is short—two to six hours—and the case fatality nil, with complete recovery in 24 to 48 hours. A period of incubation is necessary for toxin formation, and in outbreaks of this type of food poisoning it is always found that a period, generally not less than eight hours, has elapsed between the preparation and the consumption of the food. This kind of food poisoning is very common, much more so in this country[10] than in Europe where food-borne Salmonella infections (see below) account for the great majority of food poisoning outbreaks.[14, 15]

In practice, the identification of an outbreak of food poisoning as staphylococcal in etiology is assumed on the basis of demonstration of the presence of very large numbers of the bacteria, most commonly hemolytic, coagulase-positive *Staph. aureus*, in the food indicated by epidemiological evidence. It is not yet possible to demonstrate the presence of enterotoxin or the toxigenicity of the bacteria by routine laboratory examination.

Whether or not bacteria other than the staphylococci form similar enterotoxic substances is open to question. The fact that the same incubation period and clinical symptoms are observed when epidemiological evidence incriminates the other bacteria noted above suggests that enterotoxin may be formed by them. Streptococci are occasionally incriminated in food poisoning outbreaks with evidence of enterotoxin formation, and Proteus and similar organisms have been found under much the same circumstances. It appears to be established that *Cl. welchii* may also be etiologically related to food poisoning.[1, 3, 6, 12]

Food-borne Bacterial Infections. The food-borne bacterial infections are of two general kinds. The one consists of those diseases which are transmitted by a variety of vectors, of which food is but one; the symptoms are not those usually associated with food poisoning. Such, for example, are typhoid and paratyphoid fevers, dysentery, cholera, and other enteric infections. The second kind of infection is that with bacteria of the Salmonella group, in which the incubation period is short, the gastrointestinal disturbance is of short duration—a day or two—and the symptoms are typical of food poisoning. There is some crossing between the two types, for paratyphoid B bacilli (*Salmonella paratyphi* B) may produce either the typical food poisoning response or paratyphoid fever (Chap. 21).

Food-borne typhoid, dysentery, and other diseases of the first group ordinarily occur on a small scale; the great epidemics are water-borne and, to a lesser extent, milk-borne. Although limited in scope, food-borne infection plays an important part in maintaining some of these diseases in endemic form. The so-called residual typhoid, for example, which remains in spite of hygienic control of water and milk supplies is, in large part, food-borne. The employment of cooks and other food handlers who are typhoid carriers provides the opportunity for food infection and consequent transmission of the disease. Mary Mallon —"Typhoid Mary"—was one of the most notorious examples of a cook who was a typhoid carrier.

Of the Salmonella infections, one of three species is generally involved: *Sal. typhimurium* (*Sal. aertrycke*) and its varieties (*newport, stanley,* etc.), *Sal. enteritidis* and *Sal. cholerae-suis* (including Voldagsen and *paratyphi C*). *Sal. typhimurium* is the most frequently observed, while *Sal. choleraesuis* is only rarely present. The ingestion of food containing large numbers of these organisms frequently results in the typical

symptoms of food poisoning. No enterotoxic substance has been shown to be formed by these bacteria, and it is probable that actual infection takes place as indicated by the somewhat longer incubation period and the finding of the bacteria in the feces. The case fatality is variable, ranging from zero to 10 per cent. Almost any kind of food may serve to carry these microorganisms, although meats and other protein foods predominate.

Both *Sal. typhimurium* and *Sal. enteritidis* are commonly carried by rats and mice, and it is probable that in many cases these rodents are responsible for the infection of food. In others the source of infection may be a human carrier, and in many cases of meat poisoning it has been found that the meat was from a diseased animal.

As in the case of staphylococcal food poisoning, there is usually a history which includes a possible, or probable, source of contamination, and storage of the contaminated food under circumstances allowing bacterial growth. The identification of the etiologic agent found in the food is a routine laboratory procedure, involving isolation and identification by the conventional cultural and serological methods (Chap. 21).

Shellfish and Disease. Shellfish, including oysters, clams, and mussels, have been responsible for the transmission of enteric disease, especially typhoid fever and more recently infectious hepatitis, through pollution of areas in which they are grown or stored. They are commonly eaten in an uncooked or partially cooked condition which facilitates transmission of disease; it has been found that, while scalloped oysters, fried clams, and clams in chowder are practically sterilized, steamed clams, fried oysters, oysters in stew, and mussels cooked in the usual ways are not freed from coliform bacteria. The shellfish, in the course of breathing and feeding, filter large quantities of water and readily take up enteric pathogens from polluted beds or when placed in brackish waters near sewage outfalls to "fatten." Imbibed bacteria, if not ejected very soon, are passed through the gastrointestinal tract and discharged in about five hours. In the warm season the pollution of the water of a bed or storage area is highly correlated with infection of the shellfish, but during the cold months the organisms commonly show little or no infection, presumably because of greatly reduced metabolic activity. The rapidity with which bacteria are passed through shellfish suggests the possibility that they may be cleansed of infection by storage in clean or even chlorinated water. The possibility is not only feasible but practiced, four days sufficing to eliminate practically all coliform bacteria. Essentially the same methods used for the bacteriological examination of water are applied to shellfish. The United States Public Health Service has suggested that not more than 50 per cent of the 1 ml. samples of pooled shell liquor and finely chopped tissue of ten or more oysters, clams, or mussels should show coliform bacteria in the presumptive test, but the figure is a guide rather than a standard and is flexible.

Food-borne Parasitic Infections. These include the various flukes, tapeworms, echinococcus, and roundworms that infect man. In these diseases the infective stage of the parasite is often present in a food substance as a consequence of its life cycle and the mechanisms involved may be found elsewhere (Chap. 34).

REFERENCES

1. Adam, W.: Über Massenerkrankungen an Lebensmittelvergiftungen durch Pudding infolge Befalls mit *Clostridium perfringens*. Desinfektion u. Gesundheitwesen, 1958, **50**:25–26.
2. American Public Health Association: Standard Methods for the Examination of Dairy Products. American Public Health Association, New York. 11th ed., 1960.
3. Beck, A., Foxell, A. W. H., and Turner, W. C.: An outbreak of food-poisoning due to *Cl. welchii*. Brit. Med. J., 1954, **ii**:686–687.
4. Dack, G. M.: Food Poisoning. University of Chicago Press. 3rd ed., 1956.
5. Dewberry, E. B.: Food Poisoning: Food-borne Infection and Intoxication. Nature, History, and Causation. Measures for Prevention and Control. Leonard Hill, London. 4th ed., 1959.
6. Dickie, G. G., and Smith, J.: Food poisoning associated with heat-resistant *Cl. welchii* type 6. Health Bull., 1957, **15**:81–82.

7. Dublin, T. D., Rogers, E. F. H., Perkins, J. E., and Graves, F. W.: Milk-borne outbreaks due to serologically typed hemolytic streptococci. Amer. J. Pub. Health, 1943, **33:**157–166.
8. Foster, E. M., et al.: Dairy Microbiology. Prentice-Hall, Englewood Cliffs, New Jersey. 1957.
9. Hammar, B. W., and Babel, F. J.: Dairy Bacteriology. John Wiley and Sons, New York. 4th ed., 1957.
10. Hodge, B. E.: Control of staphylococcal food poisoning. Pub. Health Repts., 1960, **75:**355–361.
11. Jezeski, J. J.: The microbiology of dairy products. Ann. Rev. Microbiol., 1954, **8:**429–448.
12. Kische, F. E., and Elek, S. D.: Experimental food poisoning by *Clostridium welchii.* Lancet, 1957, **ii:**71–74.
13. Nilsson, G.: Reducing properties of normal and abnormal milk and their importance in bacteriological grading of milk. Bact. Rev., 1959, **23:**41–47.
14. Public Health Laboratory Service: Food poisoning in England and Wales, 1959. Monthly Bull. Ministry of Health and Pub. Health Lab. Service, 1960, **19:**224–237.
15. Seeliger, H. P. R.: Food-borne infections and intoxications in Europe. Bull. W.H.O., 1960, **22:**469–484.
16. Smith, J.: Milk-borne disease. J. Dairy Res., 1957, **24:**121–133.
17. Symposium: Recent advances in the bacteriology of milk and milk products. J. Appl. Bact., 1955, **18:**322–395.
18. Symposium: Food poisoning: The scope of the problem. Practitioner, 1955, **174:**645–696.

Chapter 13

IMMUNITY

Antigens, Antibodies, and the Antigen-Antibody Reaction[4, 6, 18, 21, 38, 59, 61]

It has been common knowledge for many years that recovery from certain of the infectious diseases is accompanied by the development of an enhanced resistance, and second attacks of a disease once overcome are not common. This enhanced resistance, or immunity, is specific, *i.e.*, an individual immune to one disease may be no more than ordinarily resistant to others, and is variable from one disease to another, some giving rise to a "solid" immunity of long duration while in others the immunity is imperfect or partial and transient. The specific immune state supplements the complex of factors which make up nonspecific resistance to infection, and even a solid immunity may be broken down by fatigue, malnutrition, and similar factors which are not consistent with a state of physiological well-being.

Specific immunity is a consequence of the reaction of the host to the microorganism and/or its products and provides the basis for prophylaxis of disease or infection. Its development during the course of disease is the primary determining factor in the outcome of the infection.

Historically, immunity, or immunology, was concerned primarily with disease, but it became clear that the basic principles have general biological application that has as yet been limited. Immunological specificity appears to be intimately related to the basis of biological individuality, and makes possible a precise and sensitive characterization of the larger molecular constituents of protoplasm. As such, immunological methods become unique analytical methods applicable to problems such as protein synthesis, blood and other tissue incompatibilities, and phylogenetic relationships, as well as to certain kinds of noninfectious disease or manifestations of disease such as the hypersensitivities.

Here the subject material of immunology will be considered under the general heads of antigens, antibodies, the antigen-antibody reactions, and effective immunity to disease. This differentiation is somewhat artificial and analogous to the separation of virulence and resistance. Antigenicity is demonstrable only by the immune response, and, conversely, antibody is formed in response to the antigenic stimulus.

ANTIGENS

An antigen is customarily defined as a substance which, when introduced into the tissues of an animal, provokes an immune response, demonstrable after a latent period by the appearance of antibody in the blood and other body fluids which reacts specifically with the antigen, by a specific immunity to an infectious disease when the antigen is a pathogenic microorganism and/or its products, or by an increased reactivity, or hypersensitivity, to the antigen.

This definition is that of a so-called complete antigen which both stimulates antibody production and reacts with the antibody so formed. Other substances have a more limited antigenicity in that, while they react specifically with antibody, they are unable to stimulate antibody formation. These substances are partial antigens or haptenes, and when they are found in microorganisms after destruction or removal of complete antigenic substances, they are called residue antigens.

The partial antigens, or haptenes, are, in turn, subdivided into two groups by some workers. One of these is made up of the haptenes which react with antibody *in vitro* to give the usual serological reactions (Chap. 14), and the other includes those which react with antibody, but without overt evidence of the reaction, and the antigen-antibody reaction is demonstrable only indirectly as an interference or inhibition phenomenon. This last, as will appear, is a consequence of the structure of the haptene and has no relation to its antigenic specificity.

Relative Antigenicity. The property of complete antigenicity is variable in that some substances are "good" antigens and give a marked immune response, while others are "poor" antigens and stimulate only a low-grade immune response characterized by, for instance, a relatively ineffective and transient immunity or small amounts of demonstrable antibody, etc. Substances such as ovalbumin, serum globulin, and diphtheria and tetanus toxoids are good antigens, but hemoglobin and nucleoproteins and their constituent histones are poor antigens.

Relative antigenicity must also be evaluated in terms of the animal species in which the immune response is provoked and the way in which the response is assayed; in fact, in some instances it may be difficult to demonstrate antigenicity, or lack of antigenicity, because of variation in the immune response of the test animal. In general, the rabbit and chicken are good producers of precipitating and agglutinating antibodies, but there are differences between the two with respect to the kind of antigen, *e.g.*, the antigenicity of hemoglobin is more readily demonstrable in the chicken than in the rabbit. In contrast, the guinea pig and the rat are not good producers of these antibodies, but the guinea pig is readily sensitized to anaphylactic shock by extremely minute amounts of antigen, and the rat gives a good immune response assayed as protective antibody. Similarly, certain of the high molecular weight polysaccharides, such as the pneumococcal polysaccharides, are antigenic in man and in the mouse, but not in the guinea pig or rabbit. Relative antigenicity, then, eludes precise definition.

Adjuvants.[23] A good immune response requires exposure of the antibody-forming mechanisms to the antigen for an appreciable time, even though the actual synthesis of antibody may be very rapid, and adequate amounts of antigen are required. In the first instance, a single inoculation of an antigen that is rapidly dispersed in the tissues and eliminated elicits only a minimal immune response, and the same amount of antigen is much more effective when given in successive inoculations. In the second, the degree of the immune response is directly related in a limited way to the amount of antigen administered; *e.g.*, the relation between the amount of diphtheria toxoid administered and the

antitoxin titer produced is linear over a limited range.

When the antigen is particulate or in a relatively insoluble form it tends to remain in the tissues for longer periods and so provides a more prolonged antigenic stimulus. Killed bacteria are usually good antigens, and living attentuated microorganisms (Chap. 7) often produce an actual infection in which the amount of antigen is increased over that inoculated and the antigen persists to give a prolonged stimulus. The antigenicity of soluble antigens, or even bacterial vaccines, may be enhanced by the use of adjuvants which precipitate or coprecipitate the antigen in a relatively insoluble form, or otherwise delay its absorption. An antigen depot is established, often at the site of inoculation, from which antigen is slowly dispersed to provide a prolonged antigenic stimulus. A variety of substances have been used as adjuvants, most commonly alum, calcium phosphate, and water-in-oil emulsion containing the antigen. The effectiveness of the last is increased by the inclusion of killed acid-fast bacilli such as tubercle bacilli or related saprophytic forms—the Freund adjuvant. Antigenicity is appreciably, or even markedly, enhanced by such adjuvants. For example, two inoculations of alum-precipitated diphtheria toxoid will give an immune response as good as that produced by three inoculations of the fluid toxoid, and the antigenicity of a poor antigen may be demonstrable when an adjuvant is used.

While a prolonged antigenic stimulus is undoubtedly a major factor in the effect of adjuvants on antigenicity, others are involved also. There is, for instance, reason to believe that the immune response is enhanced by an irritant effect of the antigen or adjuvant to give an inflammatory reaction and accentuation of the response of the antibody-forming mechanisms. This is the rationale behind the so-called nonspecific protein therapy of disease in which substances such as typhoid vaccine are administered. Still other factors are involved also, and the nature of the enhancement of antigenicity by adjuvants is not fully understood.

PROPERTIES OF ANTIGENS

Antigenicity may be further characterized as the kinds of substances that are antigenic, *i.e.*, the properties associated with antigenicity. These are, in general, of three kinds. First, antigens are substances of natural origin that are of relatively large molecular size; second, they are digestible, at least to a certain extent, by enzymes present in the body tissues; and third, they are foreign, or contain structures foreign, to the antibody-producing mechanism of the test animal.

Size and Nature. Complete antigens are usually naturally occurring proteins containing a full complement of amino acids, and certain high molecular weight polysaccharides of natural origin may also function as antigens. It is possible that high molecular weight polymers may be antigenic, but with the possible exception of polyvinyl pyrrolidone[46] none has been found to be antigenic. Large molecular size invariably accompanies the ability to stimulate antibody formation, though all large molecules are not antigenic, and it is probable that molecular weights of 10,000 or greater are essential to antigenicity. There is, in fact, some rough correlation between relative antigenicity and molecular weight in that antigens having molecular weights of less than about 40,000, such as lysozyme, protamines, histones, and insulin, are usually poor antigens. Consistent with this, the antigenicity of a protein disappears rapidly on enzymatic hydrolysis, and proteoses and polypeptides are not antigenic.

A number of derived proteins, notably gelatin, are not antigenic, or only feebly antigenic in conjugated form, even though of high molecular weight. Gelatin is deficient in aromatic amino acids, and it has been supposed that an adequate content of aromatic radicals may be associated with antigenicity, but there is little definitive evidence for this.

Conjugation of an antigenic protein with another substance usually does not impair its antigenicity, and may even enhance it, but often alters its specificity

(see below). In fact, many naturally occurring antigens are complexes of protein with other substances, commonly polysaccharides, and good antigens, such as ovalbumin and globulin, often contain carbohydrate as an integral part of the molecule. There are a number of exceptions among naturally occurring complexes; the nucleoproteins are not better antigens than the histone moiety, and a number of glycoproteins are poor antigens.

Degradation. The susceptibility of the antigen molecule to enzymatic degradation in the tissues appears to be a highly significant element of antigenicity. Particulate antigens, such as microorganisms, are phagocytosed and broken down both physically and chemically, and soluble protein antigens are readily metabolized. Not only is it not necessary that the intact antigen molecule persist as such, but there is reason to believe that the immediate antigenic stimulus is, in fact, an active fragment of the antigen which affects directly the antibody-forming mechanism. Such active fragments have not, however, been detected in the products of enzymatic degradation of antigens *in vitro*. The effect of an antigen depot produced by the inclusion of an adjuvant with the antigen is not one of preventing degradation, but rather of reducing the rate at which degradation occurs to provide a continuing source of antigenic stimulus over a longer time and more efficient utilization of antigen.

The significance of antigen degradation is illustrated in another way in the case of pneumococcal polysaccharide. As indicated above, this substance is antigenic in the mouse, but it is metabolized only very slowly by the tissues and is excreted largely unchanged in the urine. As a consequence, an apparently anomalous situation occurs in which the polysaccharide stimulates antibody formation only when given in minute amounts. When larger amounts are administered, the antigen accumulates in the tissues where it is directly demonstrable, blocking the "detoxifying" mechanisms. The animal becomes refractory and will no longer form antibody, a state that has been called "immunological paralysis" by Felton.

Alien Nature. The third essential prerequisite of antigenicity is that the antigen be foreign and distinguishable as "not-self" by the antibody-forming mechanism.[15] As pointed out elsewhere (Chap. 9), the distinction between self and not-self is not made by embryonic or fetal tissue, and the property of making the distinction is usually not fully established until some time post partum, so that the animal immature in this respect is unable to form antibody. Otherwise the animal reacts to the foreign nature of the antigenic substance by the formation of antibody except in the extremely rare condition of hypogammaglobulinemia.[25]

The foreign quality of antigens may be of varying degree, ranging from that of antigenic substances of phylogenetic origin widely different from the recipient animal, to isophile antigens or iso-antigens occurring among individuals of the same species and within single individuals. The alien nature of antigens is reflected in the specificity of the antigen-antibody reaction, and widely different antigens appear to be quite unrelated immunologically, while closely related antigens show cross-reaction of varying degree with appropriate heterologous antibody and behave as poor antigens when they are closely related to constituent antigens of the recipient animal.

ANTIGENIC SPECIFICITY

In general, antigenic substances from one species of animal stimulate antibody formation in another animal species, and antigens of plant origin are antigenic in animals. The relation between antigen and antibody is highly specific, as indicated above, in that antibody produced in response to the antigenic stimulus reacts specifically with the homologous antigen, and the antigen reacts specifically with homologous antibody. In a sense the serological methods (Chap.

14) used to demonstrate the antigen-antibody reaction are analytical methods, sensitive, roughly quantitative, and highly specific for components of protoplasm which are antigenic.

Species Specificity. Serum proteins are among the most commonly used antigens and may be used to illustrate specificity. Serum proteins from the horse, sheep, chicken, man, etc., are antigenic in the rabbit, while rabbit serum protein is not. Similarly, rabbit, horse, etc., serum protein is antigenic in the chicken, and the homologous chicken serum protein is not. Specificity is also expressed in the reaction of antigen with antibody. An antiserum to, for instance, horse serum protein prepared in the rabbit will react *in vitro* only with homologous horse serum protein antigen and will not react with serum protein from the chicken, man, cattle, etc. This kind of antigenic specificity is commonly referred to as species specificity although it obviously extends beyond the limits of species, through genera, and kingdoms, if plant antigens are considered also.

On the other hand, specificity is less sharp between antigens from closely related sources in that cross-reactions occur, *i.e.*, antiserum to chicken serum protein will also react with pigeon serum protein; antiserum to chicken egg albumin will react with duck egg albumin; antiserum to sheep serum globulin with beef serum globulin, etc.; but the heterologous reaction is weaker than that to the homologous antigen in that it occurs to lower titer than with the homologous antigen. Similar immunological relations occur among plant proteins as, for example, among seed proteins.

Phylogeny. It is a matter of no small interest that the immunological relationships among living organisms parallel their phylogenetic relationships based on a comparative anatomy.[35, 52] Among the primates, for example, it was shown by Nuttall at the turn of the century that the Simiidae (gorilla, chimpanzee, orangutan) are very closely related to man in the antigenic specificity of blood proteins; the old world monkeys (Cercopithecidae) are not quite so closely related; the Hapalidae (marmosets) are still more distantly related; and the lemurs, which are Lemuroidae rather than Anthropoidae, are unrelated to man. Similar relationships occur among other phylogenetic groups, plants as well as animals. It would appear to, and does, follow that antigenic specificity is genetically determined, and this is readily demonstrable within fertility groups; *e.g.*, races of experimental forms such as Drosophila, Paramecium, fowl and other birds may be differentiated on this basis with segregation of antigenic specificities, and the immunological method has become a powerful tool in genetic studies as a measure of individuality. Genetically determined antigenic specificity is of practical significance in man in connection with blood groups and the incompatibilities observed in tissue grafts (see isophile antigens below).

Antigenic specificity has practical application for forensic purposes in the identification of blood protein in blood stains in criminal inquiries and in other areas such as the detection of adulteration of beef with horse meat, etc., and is fully acceptable as legal evidence. For such purposes it is essential that the antiserum used for the identification of unknown antigen be highly specific and without significant cross-reaction. The identity of the antigen may be suggested by relative titers with respect to the homologous antigen, but the specificity of the reaction may be established by absorption of antibody reacting with the heterologous antigen (see below) in some circumstances, or by the choice of animal immunized. For example, it is easy to establish that a blood stain is fowl blood rather than human blood, but the antiserum to human blood will react with the blood of some monkeys as indicated above. Cross-reactions do not occur if the antiserum is prepared in the animal whose blood antigens show cross-reaction, *viz.*, while antiserum to sheep serum globulin prepared in the rabbit will react also with beef serum globulin, if the antiserum is prepared in cattle there will be no cross-reaction.

Isophile Antigens. Antigenic specifi-

city, then, is at least a part of the basis of biological individuality, and its significance in this respect is underscored by the occurrence of antigenic differences between individuals of the same species alluded to above, the isophile antigens, *i.e.*, isophile antigens are those which occur within the species, but not in all individuals of the species. The most important of these are those characterizing the blood groups and those concerned in the reaction to tissue grafts.

Blood groups.[22, 37, 50, 51, 58, 81, 82] It was found by Landsteiner in 1901 that incompatibilities observed in human blood transfusions have an immunological basis, and that human blood occurs in four immunological groups representing the possible combinations of two erythrocyte antigens. In the international system of nomenclature the antigens are designated A and B, and the combinations A, B, AB, and O, the last lacking both antigens. Antibodies corresponding to these antigens occur in the serum of individuals lacking the homologous antigen, *i.e.*, the antigen and corresponding antibody do not co-exist in the same blood. The serum of the individual having type O blood contains both antibodies, ab, that of the individual having type A erythrocyte antigen, the antibody b, etc. The A antigen is further subdivided into two antigens, A_1 and A_2; the type A blood, then, may be A_1A_1, A_2A_2, A_1A_2, A_1O, or A_2O, and so on in combinations with the B antigens in type AB.

Many additional erythrocyte antigens have been described also. Two designated M and N occur separately and in combination with one another, and with the A and B antigens to give twelve antigenic types of erythrocyte, neglecting the subdivision of A. Another group of erythrocyte antigens, the Rh antigens, so-called because the first of the series was found by Landsteiner and Wiener in the rhesus monkey, also occurs. Antigens of this group are related to erythroblastosis fetalis, a hemolytic disease of newborn infants due to immunization of the mother, who lacks the antigen, by antigen occurring in the erythrocytes of the child by paternal inheritance, and destruction of the infant's red cells by the antibody. The original Rh antigen is designated Rh_0; rh′ and rh″ are two additional antigens; and Rh_1 and Rh_2 are complexes containing Rh_0 and rh′ or rh″ respectively. Still additional antigens are associated with this group, *viz.*, hr′ is the antigen found in cells lacking the rh′ antigen, etc.

Still other blood group antigens have been described to give nine systems including the three noted above, AB, MN, and Rh, and it is probable that still other systems will be defined to include antigens which are not fitted into these groups.

Isophile erythrocyte antigens and the occurrence of blood groups are by no means confined to man and have been described in various lower animals, including rats, cattle, rabbits, and fish.[73]

There seems to be little doubt that the antigenic specificities of the blood group antigens are genetically determined, probably on a one-to-one relation between gene and antigen, and appear to combine in a simple Mendelian way. So the frequency of blood groups can be regarded as the phenotypic expression of human genotypes and has had valuable application in studies in human genetics. It has been found that there is appreciable segregation of such gene frequencies in subgroups or races of man. For example, 83 per cent of Eskimos have type M antigen in contrast with 57 per cent for Arabs, 28 per cent for English, and 2 per cent for Australian aborigines, and the frequency of type O blood varies from 85 per cent in American Indians, through 47 per cent for English, to 32 per cent for Russians. This subject is treated in detail by Boyd.[5]

Another application of the patterns of inheritance of the human blood group antigens is in forensic medicine in connection with disputed paternity. Comparison of the blood group antigens of mother, child, and possible father can show that a given individual could not have been the father, but cannot establish paternity.

The homograft reaction.[60, 64, 70, 72] When tissue is transplanted from one

part of the body of a mammal to another, providing that it has not been injured in the process, the transplant, or graft, becomes vascularized, grows, and establishes itself permanently. Such a tissue transplant is an autograft, *i.e.*, the donor and recipient are the same individual.

When a similar transplant is made from one individual to another of the same species, the grafted tissue also becomes vascularized, but after a few days or a week the graft tissue is invaded by lymphocytes and plasma cells, the blood vessels become dilated and engorged, the graft begins to be destroyed, and after two or three weeks destruction is complete and the graft is sloughed off. This kind of a graft is a homograft, and its rejection in the kind of reaction just described is the homograft reaction. If a second attempt is made to implant a homograft from the same donor, the reaction is accelerated, and the graft rejected even more rapidly.

A third kind of tissue graft is the heterograft in which the donor and recipient are of different species. Reaction to the heterograft is even more violent than to the homograft, and the graft is rejected.

The homograft reaction is the consequence of an immunological response to the presence of isophile antigens in the donor tissue, but not present in, and therefore foreign to, the recipient. This effective immune response appears to be largely a hypersensitivity of the delayed type (Chap. 15). Conversely, the graft tissue responds immunologically against the tissues of the recipient,[3] but it is not clear to what extent this affects the rejection.

The isophile antigens concerned are, like the blood group antigens, genetically determined. This was shown in 1927 by the work of Loeb and Wright who found that homografts between individuals of strains of guinea pigs so highly inbred as to be practically identical genetically took as well, or almost as well, as autografts. When tissue transplants were made between individuals of different inbred strains, they behaved as homografts. Further, the F_1 generation hybrids of such inbred strains accepted tissue grafts from either parent, but transplants from a hybrid to either parent behaved as homografts, indicating that neither parent contained relevant antigens foreign to the hybrid, but the hybrid offspring contained relevant antigens foreign to each parent which were contributed by the other parent. Human individuals are too heterogeneous genetically to behave in this way, but homografts between identical twins behave as if they were autografts.

The question of tissue incompatibility has been studied at some length in the mouse, and the genetic determinants of the relevant antigenicities are known as histocompatibility genes. A number of these have been described and designated H-1, H-2, etc.; of these H-2 has been the most intensively studied.[29, 65]

Prior inoculation with a suspension of living tissue homogenate accentuates the homograft reaction just as does previous tissue transplant, but it has been found that prior inoculation with a brei of killed tissue gives a variable, but sometimes enhancing, effect favoring the growth of a tumor homograft and possibly also of normal tissue homografts. The mechanism of this enhancing effect is obscure. Some workers believe that the killed tissue induces a humoral (circulating antibody) rather than a cellular (Chap. 15) immune response which favors survival of the graft by walling it off, while the living tissue gives a primarily cellular immunity that results in acceleration of destruction of the graft tissue. In this view, the humoral response, if it can be this sharply differentiated, would serve to isolate the graft from the antibody-forming mechanism in the same way that organ-specific and other iso-antigens are isolated.

Iso-antigens. In addition to isophile antigens discontinuously distributed among individuals of the same species, the individual also contains antigenic substances to which he will give an immunological response. These are iso-antigens.

The apparent contradiction is resolved by a more precise definition of the alien

nature that is a prerequisite to antigenicity by further specifying, as indicated earlier, that the antigen is foreign to the antibody-producing mechanism of the animal. Thus substances associated with reticular tissues or highly specialized cells and organs may be effectively isolated from the antibody-forming mechanism and retain sufficient alien nature to function as iso-antigens. There are a number of examples of such iso-antigens. It has long been known, for instance, that thyroglobulin is antigenic within the species,[83] that lactating animals may be able to produce antibody to their own caseins, and that viper serum will protect against the venom of the snake as effectively as the best immune serums. Further, certain organs and tissues contain antigens, which may or may not be organ- or tissue-specific (see below) but which function as iso-antigens.

Auto-immune disease.[10, 11, 14, 71] Under circumstances which are not as yet well understood, an individual may form antibody against his own tissues, resulting in auto-immune disease or collagen disease. The latter term indicates the common involvement of the connective tissue, and disease may take a number of forms, including hemolytic anemia, disseminated lupus erythematosus, idiopathic Addison's disease, Hashimoto's disease (chronic thyroiditis), and possibly also rheumatoid arthritis.

Heterogenetic Antigens. While antigenic substances are, as described above, usually sharply specific for the species or even for the individual, certain antigens occur in different, often widely separated, species, that have the same or closely related specificities. Of these antigens the α- and β-crystalline of the lens protein of the eye, the Forssman antigen, and the organ- or tissue-specific antigens are among the best known.

Forssman antigen. The most extensively studied of the heterogenetic antigens is the common, or closely related, antigenic specificity which is named after its discoverer, Forssman. It is sometimes known as heterophile antigen although it is only one of a group of these substances.

Forssman antigen is present in a variety of phylogenetically unrelated organisms including dogs, cats, sheep, horses, certain fish eggs, turtles, and toads, and certain bacteria including strains of enteric bacilli and pneumococcus, and some varieties of corn (maize). It is not found in rodents except the guinea pig and hamster, in rabbits, frogs, hogs, and most primates. When the antigen is present, it is usually found in tissues and organs rather than in the blood, and in the sheep is found in the erythrocyte but not in other cells. Application of the fluorescent antibody technique (Chap. 14) has shown that the antigen occurs in the endothelium and adventitial connective tissues of blood vessels in organs, with some species differences such as its presence in the collecting tubules of the kidney of the guinea pig and chicken, but not in these tissues of the mouse, dog, and cat.[76]

For a time it was thought that the Forssman antigen is a lipid since it could be extracted from tissues containing it in ethanol, but it now seems clear that its antigenic specificity is a function of a carbohydrate group present in the antigen. Its specificity is related to that of the human blood group A substance, and both are related to a fraction of hog gastric mucin, probably through content of similar carbohydrate groups.

The apparently random distribution of Forssman antigenicity is of practical significance in the interpretation of certain immunological reactions. For instance, the hemolytic antibody that lyses the sheep erythrocyte is usually Forssman antibody; in fact, Forssman's original observation was the lysis of sheep red cells by antiserums to guinea pig tissue prepared in the rabbit.

Organ-specific antigens. Still other antigens whose specificity crosses species lines are those associated with certain organs and tissues. Kidney tissue, for example, contains antigenicity common to this organ from different animal species as well as species-specific antigens. In at least some instances antigenicity is associated with the heavy protein frac-

tion separable from tissue homogenates by high speed centrifugation. They may be in part mitochrondria or, in the case of virus-infected tissues, the viral agent, and from the latter point of view the normal tissue components represent artifacts.[27]

Immunological studies[24, 31] have shown that the heavy proteins from various organs have three kinds of specificity, *viz.*, species specificity, organ specificity related to the same organ in other species, and specificity for the organ of the one species. By the use of labeled antibody as by iodination with I^{131} or diazotization with S^{35}-containing compounds, it is possible to show that antibodies to the tissue-specific antigens localize in those tissues, usually in the vascular bed, and tissue-specific lung, liver, and kidney antigens may be demonstrated in this way.[57]

Antibodies to such organ- and tissue-specific antigens may have a cytotoxic effect on the tissue in the intact animal. The kidney has been the most thoroughly studied in this connection, and heterologous nephrotoxic antibody has been found to produce nephritis with gross changes in the kidney, impairment of function as indicated by proteinuria, and histopathology of the glomerular epithelium.[1, 43] Similarly, continued inoculation with vaccines, such as rabies vaccine, containing brain and cord substance may affect the central nervous system by stimulating the formation of antibody which reacts with nerve tissue, and it has been shown that acute disseminated encephalomyelitis may be produced experimentally by the inoculation of homologous brain tissue with adjuvants.[79]

Acquired Tolerance.[19, 28, 47, 63, 68] It was pointed out earlier that embryonic tissue does not form antibody, apparently because it is unable to distinguish the foreign nature of an antigenic substance not normally present in that tissue. It is a matter of particular interest that antigenic substances, even though they may be foreign in the sense that they do not occur normally in the individual, may be accepted as self when present in the embryonic tissue even after the capacity to make the distinction between self and not-self is developed. This acceptance, and consequent failure to form antibody to that antigenic substance even though the antigenicity of other foreign substances is unimpaired, has been called an acquired tolerance.

The naturally occurring phenomenon was demonstrated in 1945 by Owen[13] who found that sibling cattle twins had the same blood types much more often than expected on the assumption of a random segregation of the relevant genes. This was found to be a consequence of a common, though not invariable, occurrence of a common blood supply to the twins, resulting in a natural graft of blood-forming tissue to produce a double blood type or blood type chimera. Having been implanted in the fetal organism, and thereafter continuously present, this tissue was not subsequently recognized as not-self, and an acquired tolerance, or lack of immune response to the otherwise foreign antigen, persisted after the capability of giving an immune response had developed.

The same phenomenon is demonstrable also in the response to tissue transplants, twin cattle siblings reacting to the homograft as if it were an autograft, presumably as a consequence of a "graft" *in utero* of epithelial tissue. The phenomenon is demonstrable experimentally by inoculating mice *in utero* with epithelial tissue and showing that adults of mice so treated respond to homografts as if they were autografts while untreated mice give the homograft reaction. Similarly, animals inoculated with antigens such as toxoids subsequently show a marked impairment of ability to form antitoxin although the immune response to other antigens is not affected.

THE CHEMICAL BASIS OF SPECIFICITY[40]

A large body of sound experimental evidence has established the fact that the specificity of antigens is determined

by their chemical composition. Early experimental investigation of a wide variety of antigenic proteins has shown that immunologically identical proteins are, so far as can be determined, identical in composition; that antigenic proteins differing from one another in composition are also immunologically distinct; and that antigens showing some degree of cross-reaction are closely related in chemical structure. Conclusive evidence that immunological specificity is a property of certain atomic and molecular arrangements has been obtained through the study of altered specificity and artificial antigens.

Altered Specificity. The specificity of an antigenic protein may be altered by heating, partial denaturation, treatment with formaldehyde, etc., in such a way that part of the original specificity is lost but species specificity remains although somewhat broadened. Heated rabbit serum, for instance, behaves as a foreign protein in the rabbit, and the antibodies produced will not react with unheated rabbit serum. Retention of species specificity is indicated by the fact that antibodies to the heated serum react with the homologous antigen and to only a slight degree with heated serums from other animals. Such heated proteins are known as *coctoproteins*. Treatment of a protein with iodine, nitric acid, or nitrous acid (the last to give the intermediate diazo compounds), on the other hand, alters the specificity of the antigen so profoundly that species specificity is largely destroyed. Nitrated rabbit serum, for example, acts as a foreign protein to the rabbit, and the antibodies developed will react not only with the homologous antigen but with nitrated protein derived from other animal species or even from plants. Since iodine, nitrate, and similar substances react with the protein by substitution in the benzene rings of the aromatic amino acids, these findings have been interpreted as indicating that species specificity is associated with aromatic radicals and their arrangement within the protein molecule, a conclusion which, as will appear, is no longer justified.

Largely through the work of Landsteiner and his colleagues in the preparation and study of a large series of artificial antigens, some aspects of the phenomenon of immunological specificity are now relatively well understood. It has been found that the species specificity of antigens may be altered in ways other than attacking the aromatic radicals. Acetylation with acetic anhydride, esterification with ethyl alcohol, and methylation with diazomethane, all changes in the salt-forming groups of the protein molecule, result in a change in specificity similar to that obtained by treatment with iodine or nitric acid. It is of particular interest that such a marked effect on specificity may be produced by the addition of the small acetyl, ethyl, or methyl groups to the large protein molecule. Even phosphorylation has been found to alter the immunological properties of egg albumin.

Artificial antigens.[13] The influence of the addition of relatively simple organic radicals on the immunological specificity of antigenic proteins has been further elucidated through the study of the immunological behavior of a variety of compounds prepared from protein and the diazonium derivatives of a number of methyl, chlor, brom, and nitro substitution products of aniline and *o*-, *m*-, and *p*-aminobenzenesulfonic acid as well as the parent compounds, and others such as *p*-aminophenylarsenic acid, *o*-, *m*-, and *p*-aminocinnamic acid. From these and other experiments it is apparent that the *immunological behavior of antigens is specifically modified by a relatively small part of the large protein molecule, and the specificity of the antigen is determined by the chemical structure of this part, cross-reactions occurring between antigens whose determinant portions are closely related aromatic groups.* The spatial arrangement in the determinative groups, as well as their nature, is reflected in immunological behavior; the position, *ortho*, *meta*, or *para*, of substituted acid groups in the aromatic radical produces differences in specificity, the stereoisomers of tartaric acid and *p*-aminobenzoylphenylacetic

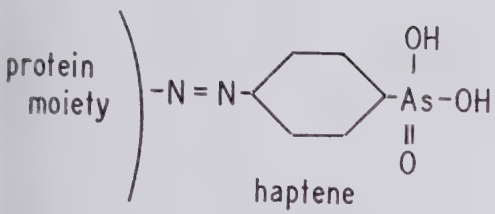


Figure 63. An example of a conjugated protein antigen prepared by coupling *p*-azophenyl arsenilic acid with a protein by diazotization.

acid yield immunologically distinct antigens when coupled with protein, and the position of amino acids in peptide-azoproteins is a factor in determining immunological specificity. In the case of artificial antigens, a minimal number of determinant groups must be added to the protein moiety to alter its specificity, 10 to 20, the number depending upon the size of the molecule, but not all are functional in the combination with homologous antibody (see below).

If it be assumed that the amino acids comprising the peptide chains of the protein molecule are arranged in regular and recurring order and proportion and, consistent with present theory of protein structure, that these chains are folded, then it is apparent that the recurring combinations of amino acids in the folded chains will result in "nodes" of molecular structure and patterns of polar groups. It is supposed that this type of molecular structure confers immunological specificity on the protein molecule that is not combined with some other type of structure.

In any case, a relatively small portion of the surface of the antigen molecule serves to determine its immunological specificity. It might, therefore, be anticipated that a single molecular species of antigen might provoke the formation of more than one kind of antibody specificity, *i.e.*, have more than one kind of determinant. There is reason to believe that this is true, at least to a limited extent. Among the naturally occurring antigens, the pneumococcal antigen includes both the polysaccharide hapten-determined type specificity and the species specificity of the protein moiety complexed with another carbohydrate. The former is quantitatively predominant, and early during the course of immunizing inoculations the antiserum is sharply type-specific, but on continued immunization antibody to the species-specific antigenicity becomes more and more evident. It is a general observation that hyperimmune serums are often not as sharply specific as antiserums obtained by a less prolonged course of immunization. Further, while the specificity of a protein antigen may be altered as just described, antibody may be produced to the original as well as the new specificity.

It may not be possible to distinguish multiple antigenic specificities in a presumably "pure" antigen. The differentiation of antigenic specificities by the procedures of antigenic analysis (see below) is dependent upon the independent occurrence of the component specificities. Any antigen regarded as immunologically homogeneous in that it has only a single demonstrable antigenic specificity may, in fact, have multiple specificities which occur only in this particular combination and are, therefore, not differentiable.

Haptenes. The immunological function of the haptenes, or partial or residue antigens, now becomes clear in their role as determinants of immunological specificity. The relative importance of hapten-determined specificity is very great, for a large portion of naturally occurring antigenic substances are conjugated antigens consisting of a protein moiety and a haptene, and the latter is often found in the form of residue antigen in microorganisms. The significance of hapten-determined specificity in infectious disease is illustrated in the pneumococcus whose polysaccharide capsular substance provides the antigenic specificity required to produce an effective immunity to infection with these microorganisms.

Polysaccharides.[8] Of the residue antigens those of the pneumococcus have been the most intensively studied and for present purposes may be regarded as typical. These so-called *specific soluble substances* (SSS), which make up the capsule of the bacterium, have been found by Heidelberger, Avery, Goebel,

and others to be polysaccharide in nature. Of the pneumococcal type-specific polysaccharides, that of type 3 has been the most thoroughly investigated and appears to be a polymer of aldobionic acid units (in this case glucose and glucuronic acid joined through a glucoside linkage, 4-β-glucuronosidoglucose, designated as cellobiuronic acid) having a molecular weight of 1000 to 5600. The nature of the other type-specific polysaccharides is not so clear. Type 2 polysaccharide hydrolyzes to glucose; type 1 is possibly a trisaccharide made up of two uronic acid (in part galacturonic acid) molecules, a third molecule containing nitrogen, and acetyl groups whose presence is of considerable immunological significance; and type 4 yields an amino sugar on hydrolysis. That such polysaccharide haptenes are not peculiar to the pneumococcus is indicated by the isolation of similar substances from a variety of microorganisms including yeasts and other fungi and certain metazoan parasites as well as many species of bacteria.

The presence of capsular polysaccharide material confers immunological specificity upon the pneumococcus types; the rough, nonencapsulated forms are species-specific, and no longer may be differentiated into types irrespective of the immunological type of the smooth, encapsulated parent form. The species specificity manifested by the rough forms, furthermore, appears to be intimately associated with the presence of yet another polysaccharide, the so-called C polysaccharide, which is common to the rough forms and which, like type 4 polysaccharide, hydrolyzes to an amino sugar but also contains phosphoric acid. The genetic transduction of pneumococcus types, then, would appear to be an alteration in the ability of the microorganism to synthesize one or another of the type-specific polysaccharides.

The immunological specificity of other bacteria, including types A, B, and C of Friedländer's bacillus, certain of the meningococcus types, staphylococci, streptococci, and other microorganisms, is similarly associated with the presence of polysaccharide haptenes. The nature of the linkage between polysaccharide and antigenic protein in the bacterial cell is not known.

It has been shown also that an effective antigen need not contain intact polysaccharide; Goebel has prepared an azoprotein antigen from horse serum and the glucoside of cellobiuronic acid whose antiserum will protect mice against type 3 pneumococcus infection. Similar protection is afforded against type 2 infection by an antiserum to an azoprotein antigen prepared from the glucoside of a *synthetic* isomeric aldobionic acid, gentio-biuronic acid (6-β-glucuronoside-glucose).

Lipids.[80] Principally because of the apparent solubility of Forssman antigen in organic solvents and the immunological activity of certain lipoidal preparations from tubercle bacilli, it has been thought that lipids might function either as complete antigens or as haptenes. The experimental evidence, *e.g.*, immunization with mixtures of foreign serum and such substances as lecithin, cephalin, and cholesterol, has, however, been inconclusive, and it is, therefore, uncertain as yet whether or not lipids may determine the immunological specificity of antigenic proteins. A number of steroids have been found to act as haptenes when conjugated with bovine serum albumin to form a complete antigen.[2]

Drugs. An immune response, often manifested as a hypersensitivity, to low molecular weight substances, including antibacterial substances and other drugs as well as cutting oils and similar substances responsible for occupational dermatitis, occurs with some frequency. It is probable that the low molecular weight nonantigenic substance combines with protein of the recipient to function as a haptene and alter its specificity so that it becomes a foreign substance and antigenic to provoke an immune response. The antibody so formed reacts specifically with the drug or other substance functioning as a haptene, and allergic

reactions occur. Such sensitization to penicillin, for example, is observed relatively often.

THE ANTIGENIC STRUCTURE OF MICROORGANISMS[42]

The term antigen may be used loosely to mean any antigenic material or, more precisely, to mean a single antigenic substance which is immunologically pure in that it contains only a single antigenic specificity. The great majority of antigens occur in mixtures with other antigens, and microorganisms are such mixtures, containing more than one, often very many, component antigens. The microorganism, then, consists of a mosaic of independent antigens, each of which stimulates antibody formation and reacts specifically with its homologous antibody. Because of this, an antibody may be preferentially removed from an antiserum containing many kinds of antibodies by adsorption on its specific antigen to provide the basis for antigenic analysis (see below), and characterization of the microorganism by its content of differentiable antigens, *i.e.*, by its antigenic structure.

The distribution of antigens among microorganisms is analogous to that among higher forms in that individuals, or more accurately clones, may be immunologically specific, and at the same time show cross-reactions with similar forms, but some microbial antigens are heterogenetic and occur in widely separated forms. With appropriate reservations, antigenic specificity may be taken as a measure of biological individuality among microorganisms just as in higher forms, and evidence continues to accumulate that it is genetically determined also.

Differentiation of Antigens. Microbial antigens are separable into groups on the basis of chemical properties, especially resistance to heat (100° C.) denaturation, and on the basis of location in the cell structure, as well as by immunological specificity. Those associated with cell structure include the antigens present in bacterial flagella, the flagellar antigens, those present in the capsule or envelope, the capsular or envelope antigens, and those present on the cell surface proper such as certain of the type-specific antigens of the streptococci. None of these appear to be essential parts of the cell since they may be removed with no apparent effect on viability. The intracellular antigens of the cell substance proper are the so-called somatic antigens and, in some instances at least, appear to be integral parts of the cell wall. In the case of microorganisms containing endotoxins, the toxicity is intimately associated with antigenicity, and endotoxin and somatic antigen may be wholly or in part identical. In addition to these kinds of antigens, soluble antigenic substances secreted by the microbial cell, such as exotoxins, various toxic activities such as hemotoxins and cytotoxins, and enzyme or enzyme-like activities such as kinases, are used also in the immunological characterization of microorganisms.

Some of the variety of antigens that may be present have been alluded to earlier. The capsular antigens, which are usually polysaccharides, but may be polypeptides, have been described above in connection with haptene-determined specificity. The myosin-like flagellar protein, flagellin (Chap. 3), includes the heat- and alcohol-labile H antigens, so-called because their presence in enteric forms is associated with motility and a spreading (Hauch) type of growth on agar mediums. The somatic antigens of the enteric bacilli are the lipid-polysaccharide-polypeptide complexes that are the endotoxins, or lipopolysaccharides, (Chap. 9), and they are called O antigens because they are present when the microorganism is nonmotile and growth on agar mediums is not spreading (ohne Hauch). The exotoxins and other toxic activities have also been described elsewhere (Chap. 9).

Characterization. The immunological characterization of a microorganism may be relatively simple, in that one or two antigens may suffice for differentiation,

or complex and concerned with many antigens. The diphtheria bacillus is an example of simple practical differentiation and is identified by its production of immunologically specific diphtheria toxin, and other characteristics are peripheral to this one. The pneumococcus is somewhat more complex for two kinds of antigens are involved, that of the cell substance proper, and that of the type-specific capsular substance. Differentiation of streptococci makes use of four kinds of antigens, a polysaccharide group-specific antigen and at least three type-specific antigens of polysaccharide, protein, and nucleoprotein nature. The antigenic structure of the enteric bacilli is more complex. In the Salmonella group the antigens are separable into the flagellar or H antigens, and the somatic or O antigens, with the former subdivided into phase 1 and phase 2 flagellar antigens. The somatic antigens were arbitrarily numbered by Roman numerals (Arabic numbers are beginning to be used), and the flagellar antigens by Arabic numerals and by lower case letters. Using these symbols, the antigenic mosaic of a bacterium of this group may be given as an antigenic formula; the paratyphoid B bacillus, for instance, has the formula I, IV, V, XII: b — 1,2. The coliform bacilli are equally complex antigenically, containing in addition to H and O antigens, somatic surface or envelope antigens called K antigens, and the K antigens are subdivided into L, A, and B groups of antigens. The antigenic characterization of these and other microorganisms is considered in subsequent chapters devoted to more detailed consideration of individual microorganisms.

Common Antigens. The heterogenetic antigens of microorganisms are usually called common antigens, *i.e.*, are common to more than one kind of organism. The Forssman antigen is one of these and is found in some bacteria, but not others, as well as in higher forms as indicated earlier. Other common antigens are shared by microorganisms with other forms, such as the immunological relationship between type 14 pneumococcal polysaccharide and the human blood group A substance; and those of type 2 pneumococcus, type B Friedländer's bacillus, and other coliforms to certain species of yeasts and vegetable gums such as gum acacia. Other common antigens occur between microorganisms as widely separated as the cholera vibrio and Brucella and the plague bacillus and paratyphoid B bacillus. There are many such examples, and it is clear that certain antigenic specificities found in microorganisms are heterogenetic in the sense that they seem to occur at random.

Antigenic Variation. The antigenic character of microorganisms is subject to variation (Chap. 7), and this may be viewed as variability in the ability to synthesize antigenically specific substances. In bacterial variation of the S-R type followed immunologically, the microorganism may be successively "degraded" with the disappearance of antigens and the appearance of apparently "new" antigens. This has been taken by some as indicative of the occurrence of antigens in layers like the layers on an onion which may be peeled off in successive degradations to expose new antigens. With the exception of flagellar and capsular antigens, there is no substantiation for such a view. It is more probable that the effect is one of alteration in the ability of the microorganism to synthesize haptenes, and when it fails to produce a haptene, such as a carbohydrate, the antigenic specificity of the protein moiety appears as a new antigen, or a new antigen occurs as a consequence of synthesis of an altered haptene to give a different antigenic specificity to the conjugated antigen.

ANTIBODIES[67, 69]

The immunological response of an animal to the initial injection of an antigenic substance is not immediate, but after a suitable time interval or incubation period is manifested as an alteration in the properties of the blood serum with respect to the antigen. An immune serum, or antiserum, differs from normal serum in that it reacts, either *in vivo* or *in vitro*, with the homologous antigen. This property of immune serum is a consequence of the presence of *antibodies*, substances which are formed by the animal body in response to the presence of antigen in the tissues and which combine specifically with the antigen. Since antibody is found in largest amount and in most convenient form in the blood serum, the properties and nature of antibody may be considered in terms of serum antibody.

Nature of Antibody. Antibody activity is intimately associated with serum protein, and antiserums may contain 1 to 5 mg. per ml. or even more antibody protein. In general, the activity is localized in the globulin fraction of the serum protein, the first to be precipitated by sodium or ammonium sulfate or ethanol in the cold, but usually the concentration range over which precipitation occurs is not sharp. When serum globulin is salted out, as by half-saturation with ammonium sulfate, all of the antibody activity is precipitated, occurs in both the euglobulin and pseudoglobulin fractions, and usually less than 50 per cent of the total globulin, even in a hyperimmune serum, is antibody of a given specificity.

Physical properties. On electrophoretic fractionation of serum protein, the globulin is separable into three fractions on the basis of mobility, α-globulin, β-globulin, and γ-globulin in order of decreasing mobility. Antibody activity occurs almost exclusively in the γ-globulin fraction, but there are occasional exceptions in which the several antibodies produced in response to inoculation with a complex antigen may be segregated in different protein fractions. For example, in the guinea pig sensitized with tubercle bacilli, antibody to the tuberculopolysaccharide is contained in the γ-globulin fraction, while that to tuberculoprotein is found in the α-globulin and even to a certain extent in the albumin fraction.[20] Or antibodies to the antigenic complex of the sheep erythrocyte which occur in the γ-globulin fraction may be found in separable parts of that fraction. Such variation in the distribution of antibody activity may be related to certain observed differences in the stability of antibody to different antigens. For instance, preservation of H-O antiserums with 0.5 per cent phenol is undesirable because on storage there is a preferential reduction and even eventual disappearance of antibody to the O antigen. Similarly, the antibody to O antigen is markedly reduced by heating to 70° C., while that to H antigen is not.[48]

In general it has not been possible to separate antibody activity from serum globulin, using a variety of purification procedures (see below). Thus, diphtheria antitoxin has been prepared as a pure protein crystallizing in thin plates and containing 700,000 to 1,000,000 antitoxic units per g., and evidence such as this has led to the inevitable conclusion that antibody is modified serum globulin.

Examination of immune globulin by various physical methods, including osmotic pressure, diffusion, and viscosity measurements, and determination of sedimentation rates by high speed centrifugation, has shown that it has a relatively constant molecular weight of approximately 160,000 and is an asymmetrical molecule of cylindrical or cigar-like shape with a ratio of length to diameter (axial ratio) of about 8.

While this description is one of the "classic" antibody found in the 7S fraction of sedimented serum protein, a high molecular weight antibody is found also and occurs in the 19S fraction. It was originally described as formed by horses, pigs, and cattle to pneumococcus, al-

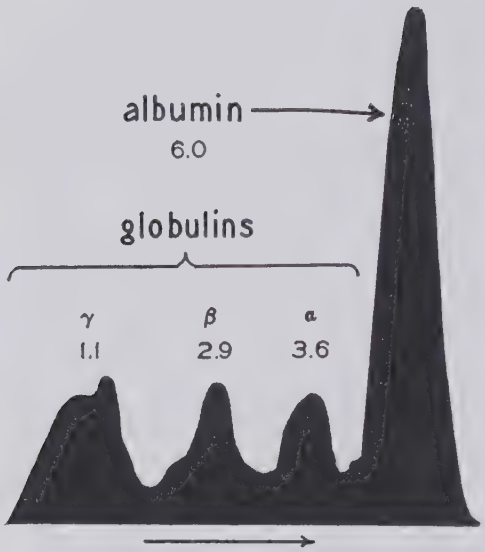


Figure 64. Serum protein fractions separable by electrophoresis. The small tip on the γ-globulin fraction is the T fraction or γ_1 fraction. The numbers give the approximate mobilities of these fractions from rabbit serum in $\mu \times 10^5$ sq. cm./sec./v. at pH 7.72 in 0.15 M NaCl and 0.02 M $^-PO_4$ at 5° C.

though these animals form antibody of the usual size to other antigens; it is now clear that such large antibody is not uncommon. It has a molecular weight of about 10^6, and the molecule is the same diameter as that of smaller antibody but is longer. The prototype of such large antibody is the Waldenström macroglobulins.[39]

Chemical properties. Immune globulin is indistinguishable from normal serum globulin by chemical analytical methods, and its properties are those of serum globulin. The activity is destroyed by denaturation with heat, alcohol, urea, by degradation by treatment with strong acids or alkalis, etc. Immune globulin may be reacted with other substances to give conjugates and, if the alteration is not extensive, antibody activity is retained. It is possible to label antibody without loss of activity by incorporation of substances such as I^{131} or fluorescent dyes for various experimental purposes.

While immune globulin is digested by proteolytic enzymes, the activity is more resistant than might be supposed in that antibody activity persists while accompanying proteins are digested sufficiently to reduce sharply their antigenicity. Enzymatic digestion of antiserums such as antitoxic serums has been used commercially for the immunological "purification" of such materials to minimize sensitization or reaction to extraneous protein, both by the addition of proteolytic enzymes and by the activation of plasmin as described elsewhere (Chap. 9). It does not necessarily follow that this relative resistance of the activity to proteolysis means that immune globulin is resistant to enzymatic digestion, but rather that antibody activity is retained by larger molecular fragments. Such active fragments of diphtheria antitoxin have been found to have an axial ratio about half that of the intact molecule.[66]

Antibody synthesis.[74] The outstanding differential characteristic of immune globulin is its property of reacting specifically with homologous antigen. Modification of normal serum globulin is such that this property is acquired, but the modification is of a sufficiently subtle nature that it is not detectable by the usual analytical methods.

It will be recalled that the basis of antigenic specificity is the chemical nature and configuration of a haptene or other small determinant portion of the antigen molecule. It may be taken to follow that the modification of serum globulin which gives it antibody activity can be inferred from the nature of antigenic specificity. The antigen-antibody complex formed when the two react with one another is held together by secondary valencies (see below) which are probably dependent upon the occurrence of one or more areas upon the surface of the antibody molecule in which the pattern of polar forces is a kind of mirror image of that determining antigenic specificity. There is no evidence that such an area is attached to the otherwise normal serum globulin. On the contrary, it seems to be built into the structure of the molecule, resulting, according to Pauling, from the way in which the peptide chain is folded, and the localized pattern of polar forces occurs in the same way as that determining

the antigenic specificity of a pure, unconjugated protein. After folding has been completed, the configuration is stabilized by the insertion of -S-S- and hydrogen bonds.

Theories of antibody formation.[7, 17] In a very real sense, in the stimulation of antibody formation, the presence of antigen acts as an inducer of the synthesis of immune globulin in a manner analogous to the function of the inducer of adaptive enzyme formation (Chap. 7). The specificity of the product of such induced synthesis is attributable to the template provided by the determinant portions of the antigen molecule. The most widely accepted theory of antibody formation was suggested by Breinl and Haurowitz and by Mudd at about the same time (1930–31). According to this theory, antigen, and more recent evidence suggests an antigenic fragment, persists for extended periods in the antibody-forming cells (Chap. 15) to provide the template against which specific antibody is formed. The striking analogy to the induction of adaptive enzyme formation has been extended in an attractively plausible theory of antibody formation by Burnet and Fenner[16] in which the initial antigenic stimulus functions as an inducer, and the enzymatic adaptation giving altered, or immune, globulin persists for an extended period after the inducer, or antigen, has disappeared.

The foregoing is known as the "instruction" kind of theory, *i.e.*, the antigenic stimulus, whether it be a fragment of an antigen or derived from an antigen, is external to the genone of the antibody-forming cell. A second kind of theory, the "selection" theory, was proposed by Jerne [36] in 1955, and has been elaborated by Burnet and others.[12, 13, 41, 75] In this kind of theory the preexistence of individual antibody-forming cells (Chap. 15) which, in the aggregate, can synthesize all possible immune globulins, is assumed. The antigenic stimulus, then, results in the preferential proliferation of those having the immunological potentiality to react with the antigen. Because of the selective effect of the antigenic stimulus, this kind of theory has been called the "clonal selection" theory. It may be considered to be a sophisticated version of the classic side-chain theory of Ehrlich (see below). The obvious objection to it is the necessity for postulating the appropriate, and essential, random somatic mutations of mesenchymal cells, presumably during the course of embryonic development, to give clones of the required specific immunological potentialities.

It is not yet possible to assay the validity of these various theories of antibody formation, but the generalities are not mutually exclusive and they may well be combined in time. The sites of antibody formation, the persistence of antigen in the cells and tissues, and similar factors are significant elements (see Chap. 15).

Rate of formation. However this may be, the formation of antibody is, in large part if not entirely, a total synthesis as indicated by the extremely rapid incorporation of labeled amino acids from the amino acid pool, with a transit time of 40 minutes at the most, and possibly considerably less, during the stage of

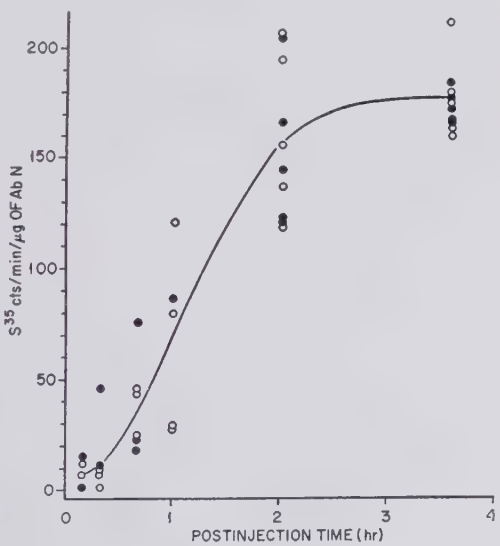


Figure 65. The rapid total synthesis of antibovine serum albumin antibody by the rabbit is shown by the rate of incorporation of S^{35}-labeled amino acids into the antibody molecule. (Taliaferro and Taliaferro.)

active antibody formation. The rate of formation of antibody is deduced from serum antibody titer after correcting for the catabolism of antibody already present whose half-life ranges from two to five days. It is usually more rapidly destroyed in smaller animals having higher metabolic rates, but exception is to be noted in the case of the human infant in which antibody persists as long or longer than in the adult in spite of a relatively higher rate of metabolism. There is also reason to believe that the rate of antibody synthesis is measured directly as copro-antibody titer since the titer of antibody in the feces is not, in contrast to serum antibody titer, cumulative, or as titer in lymph draining the popliteal nodes following inoculation of antigen in the footpad.[56]

Antibody appears in the serum of the inoculated animal only after a latent period, rises rapidly and almost exponentially and falls off somewhat before reaching a peak, and then declines relatively slowly, the time dimension of the response varying with the antigen (Chap. 15). This is the primary immune response. The latent period is regarded by some as the time required for the metabolism of the antigen, possibly to fragments which are the immediate inducers of antibody formation, and by others as a period during which a precursor is formed which subsequently reacts with antigen and is converted to antibody, and there is no doubt that a part of the latent period is required to saturate the tissues with antibody prior to the spilling over of the excess into the blood stream.

Secondary response. The secondary response is given by an animal having had a prior inoculation of the antigen, even months ago, and is characterized by a greatly reduced time required to reach peak serum titer—in the case of toxoids the time is reduced from two to four weeks to a few days—and the peak titer is often considerably higher than that reached following primary inoculation. It is obvious that the effects of the initial antigenic stimulus persist as a hyper-reactivity of the antibody-forming mechanism to the antigen, and it is for this reason that the secondary response is called the anamnestic, or remembering, reaction. The occurrence of the anamnestic reaction presents some difficulties for theories of antibody formation; in the adaptive enzyme theory, for example, the adaptive enzymes catalyzing the synthesis of immune globulin are assumed to be self-duplicating under the second antigenic stimulus.

As indicated above, Pauling[53] has suggested that actual modification of the serum globulin occurs late in the process of synthesis, after synthesis of peptide chains is partially or largely complete. There is a certain amount of evidence for this in that it has been possible to denature, or unfold, normal globulin slowly by heating gradually to 57° C. and holding at this temperature for about two weeks in the presence of 2,4,6-

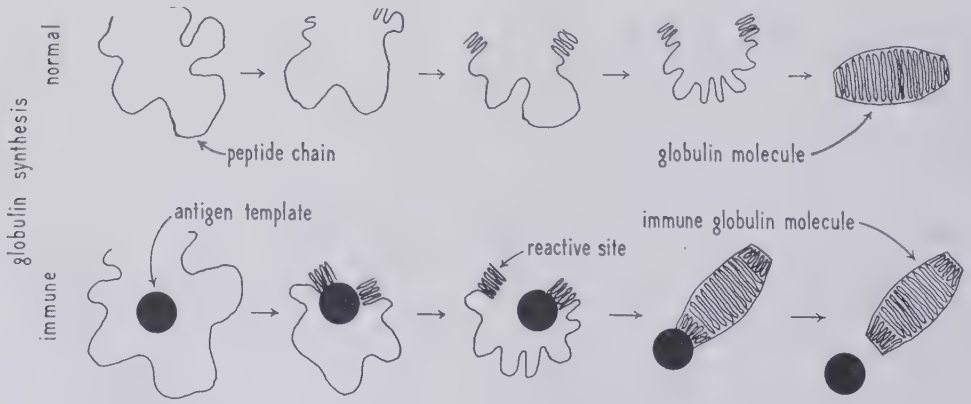


Figure 66. Diagrammatic representation of the sequence of events assumed to occur if antibody activity results from a directed folding of the peptide chain during synthesis. (After Pauling.)

tri(*p*-azophenylarsonic acid)benzene as an "antigen," and cooling slowly to renature the globulin; the renatured globulin gave some antibody-like reactions but cannot be called antibody.[54] The development of specificity as late as this in globulin synthesis is not consistent with the total, or practically total, synthesis of immune globulin from the amino acid pool. In this connection it may be pointed out that there is no evidence that normal globulin is a precursor of immune globulin, although labeled amino acids may be recycled from catabolized normal globulin and subsequently appear in immune globulin; nor is there any substantiation for the older idea that antigen is actually incorporated in the antibody molecule.

However the reactive sites are formed during the synthesis of immune globulin, there are only one or two, and probably not more than three, such areas on the surface of the molecule that are functional in that they will combine with antigen, although others may be present internally. It has been calculated that each combining site occupies a relatively small part, 1 per cent or less, of the total surface of the immune globulin molecule, and it is obvious that only a small portion of the molecule is directly concerned in the reaction with antigen. The number of such combining sites is spoken of as the valence of antibody, and, in general, antibody is monovalent or divalent in contrast with antigen which is multivalent (see below). When the antibody molecule is illustrated in diagrammatic form, the two reactive sites are usually shown at either end of the elongated molecule, but there is no reason to suppose that they occur in these positions or are even symmetrically placed.

Since the modification of globulin giving it antibody activity consists of the presence of "abnormal" localized patterns of polar forces on the surface of the molecule, it might be supposed that these areas may function as haptenes to alter the specificity of globulin as an antigen. If so, immune globulin might be antigenic in the animal producing it, and it should be possible to obtain antibody to antibody by immunization of other animals. This has not proved to be true, possibly because even if such reaction sites can function as haptenes, there are too few to alter the antigenic specificity of the globulin molecule.

Heterogeneity of Antibody. At one time it was assumed that the several antibody activities demonstrable in the various serological reactions (Chap. 14) are those of separate and distinct antibodies, that for example antitoxic and precipitating antibodies to the same antigen are different, a supposition apparently supported by discrepancies in antibody titer measured in different ways. On the other hand, the essential similarities between certain of the serological reactions, especially precipitation

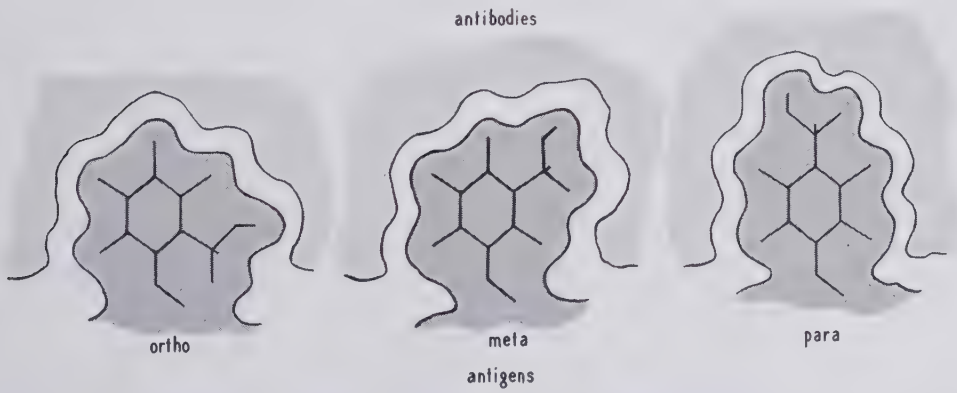


Figure 67. Diagrammatic representation of the effect of optical isomerism on antigenic specificity and the relation of the haptene to the site of antibody activity on the surface of the antibody molecule. (After Pauling.)

and agglutination, are apparent and are reinforced by observations such as the passive agglutination of particles coated with soluble antigen by precipitating antiserum and the precipitating as well as antitoxic activity of antibody to exotoxins.

The common factor in the serological reactions, and in *in vivo* titrations of antibody activity, is the union of antigen and antibody (see below), but the consequences of that union are variable and determined by the way in which the test is carried out, *e.g.*, is the antigen precipitated, is the animal protected against the toxin, etc.? Largely for physical reasons, some kinds of serological reactions require more antibody than others, thus leading to apparent differences in the amount of antibody determined. When it became generally clear that such discrepancies are, in fact, artifacts, the homogeneity of antibody to a single, immunologically pure antigen was formalized as the unitarian hypothesis. This simply states that a single antibody can produce various consequences following its union with antigen, and the different consequences observed are not evidence of the presence of more than one kind of antibody.

While this generalization holds true, it is equally clear that antibody is, in fact, heterogeneous, but in a different sense. Two kinds of heterogeneity are to be distinguished. The first is spurious in the sense that the term antigen is often used collectively. While an immunizing preparation such as typhoid vaccine is called an antigen, it is in fact a mixture of the individual antigenic components of the mosaic making up the antigenic structure of the microorganism. Or the sheep erythrocyte, which contains Forssman antigen together with several isophile antigens, is also a mixture of antigens. When an animal is immunized with such material, the immune response is multiple, a group of differentiable antibodies, each specific for one antigenic component, are produced, and the antiserum is obviously heterogeneous with respect to its antibody content.

This may assume considerable practical importance. In the first example noted above, antibody to the H antigenic complex of the typhoid bacillus is not protective and is unrelated to immunity to the disease, while the antibody to the O antigenic complex is protective and the basis of an effective immunity. Since the titer of agglutinating antibody to the H complex is commonly much higher than that to the O complex, assay of the immune response by agglutination of the typhoid bacillus by the antiserum does not accurately measure immunity to the disease. In the second example, the titration of antibody that will sensitize the red cell antigen to lysis by complement (Chap. 14) does not measure a single antibody, but rather the hemolytic antibody that happens to be present in highest titer.

Even when antibody is seemingly not heterogeneous in this respect, in that its formation is provoked by an immunologically "pure" antigen having but a single known specificity, more than one kind of antibody specificity, and therefore more than one kind of antibody, may be present in such an immune serum. It was noted earlier in connection with antigenic specificity that a single molecular species of antigen may have more than one demonstrable specificity and that what appears to be a single specificity may, in fact, be a complex antigen whose several specificities have not been found to occur independent of one another. Thus antibody to what is considered to be a single antigenic specificity may be heterogeneous, and the antiserum contains several antibodies specific for different parts of the antigen molecule. It is theoretically possible that such multiplicity may contribute to apparent differences in avidity of antibody (see below).

The second kind of heterogeneity is that of antibody to a single antigenic specificity. That some such variability occurs is already apparent from the earlier discussion of the properties of antibody. Antibody activity may, for example, occur in differentiable protein fractions. When the activity is distributed between the pseudoglobulin and

euglobulin fractions of serum protein, the former kind of antibody is soluble in distilled water while the latter is not. Or antibody activity may be a property of serum proteins of differing charge and therefore electrophoretic mobility; the occasional observation of antibody activity in fractions other than γ-globulin has been noted above, and, further, γ-globulin may be separated into two electrophoretic fractions, the γ_1 or T fraction and a slower moving γ_2 fraction, both of which may contain antibody. The extent to which such serum protein fractions are artifacts of manipulation may be questioned, but it is clear that the same antibody specificity may occur in differentiable serum protein fractions.

Incomplete antibody.[34] There is also a heterogeneity in antibody activity itself that may be divided into two kinds. The one is concerned with the number of reactive sites present on the immune globulin molecule. It was noted above that there may be one or two of these, and possibly rarely as many as three. Disregarding the last, antibody may, then, be monovalent or divalent (also see below). The former does not give the usual serological reactions and is demonstrable indirectly by its inhibiting or blocking effect on the reaction of antigen with divalent antibody and is also known as nonprecipitating, incomplete, or blocking antibody. Such monovalent antibody may be found in relatively large proportion early in immunization; naturally occuring Rh antibody is often blocking antibody, and the antibodies present in the serum of allergic persons, the so-called allergic reagins, are of this kind.

Avidity. The other kind of heterogeneity in antibody activity is a consequence of how well or how poorly the reactive sites on the surface of the immune globulin molecule are developed. The forces that bind antigen to antibody in the antigen-antibody complex are individually weak but in the aggregate provide a firm though dissociable union. When the reactive site of the antibody corresponds precisely to the determinant portion of the antigen, the maximum number of weak forces are operative, the complex is firmly bound, and the antibody is said to be highly avid. When the site is imperfectly developed, the complex is more readily dissociated, occasionally to such a degree that antigen and antibody may coexist in the same solution with only a small portion combined as the complex, and such antibody has a low avidity. Antibody is, then, heterogeneous in this respect, and antibodies of varying degrees of avidity are found in the same antiserum.

Purification of Antibody. The purification of antibody is a matter of its separation from other serum proteins, and the methods used are of two general kinds: the nonspecific chemical or physical methods; and the specific methods which make use of homologous antigen.

The nonspecific methods commonly used are the precipitation of serum globulin by salting out, usually with ammonium sulfate, or by ethanol in the cold, and by electrophoretic methods. Serum globulin is precipitated at half-saturation with ammonium sulfate, achieved by the addition of an equal volume of the saturated solution to the antiserum, and adjusting to pH 7.8. The precipitate is washed, redissolved, and the ammonium sulfate removed by dialysis. Variations of this method are used commercially to effect a partial purification of antitoxins, and preparations of this kind may be given labels such as "immune globulin solution." Immune globulin is also precipitated by ethanol. A 1:1 dilution of the antiserum is chilled to 0° C. and 50 per cent ethanol added in small increments over a period of several hours until the final concentration is 20 per cent by volume. The temperature is lowered to —5° C., and after precipitation is complete, the precipitate is removed by centrifugation and dissolved in isotonic saline. Preparations of this general nature are often called "gamma globulin." The electrophoretic separation of the antibody-containing γ-globulin from the other serum proteins can be carried out in a number of ways, but it is difficult to effect a sharp separa-

tion of γ-globulin from β-globulin. Nonspecific methods such as these separate the antibody-containing protein fraction of immune serum, but at best less than half of such preparations consist of antibody.

The specific methods of purification of antibody consist of reacting the antibody with homologous antigen to give an insoluble antigen-antibody complex which, after washing, is dissociated to give free antibody. This approach is usually practical only when the antigen is, or can be made, insoluble so that it may be removed from the mixture after dissociation. Bacteria are, of course, particulate antigens and, after combination with antibody at neutrality, the complex is dissociated at pH 3, and the bacterial antigen removed by centrifugation to leave purified antibody in solution. Soluble antigens present a more difficult problem and a number of methods have been devised. Perhaps the simplest is the adsorption of antigen on ion-exchange cellulose. Antibody is specifically removed by combination with antigen on percolation through a column, and then preferentially eluted. Or the antigen may be thiolated, and the antibody separated from the antigen-antibody precipitate by removal of the thiolated antigen.[62]

THE ANTIGEN-ANTIBODY REACTION[26, 30, 32, 33, 44, 45, 49, 55, 77, 78]

The nature of the reaction between antigen and antibody has been of great interest since the phenomenon was originally described. The first comprehensive theoretical formulation was that of Ehrlich in 1906. He made the basic assumption that specific local reactive sites or receptors for food substances and also for antigens are pre-existing on the body cells or cell structures. These receptors were regarded as having a relation to the cell structure analogous to that of side chains of complex molecules to the molecular nucleus, and this theory is known as Ehrlich's receptor theory or side-chain theory. According to this theory, when the receptors are saturated with antigen, particularly toxic antigen, the cell responds by synthesizing receptor substance in excess which then occurs free and is antibody. In order to account for the various manifestations of antigen-antibody union in the serological reactions, three kinds of receptors, of increasing complexity and designated as of the first, second and third orders, were postulated.

The combination between free receptors, or antibody, and antigen was considered by Ehrlich to be analogous to the neutralization of a strong acid by a strong base, and deviations from behavior predicted on this basis were accounted for by postulating varying degrees of avidity of antibody. This theory was slightly modified by Arrhenius in assuming that the antigen-antibody reaction is analogous to the neutralization of a weak acid by a weak base, so accounting for discrepancies such as dissociation of the antigen-antibody complex. These theories have little more than historical interest, but certain of the concepts and terminology, such as the term receptor, persist with modified meaning.

A more precise understanding of the nature of the antigen-antibody reaction began with Bordet's insistence in 1920 that it be considered as an adsorption phenomenon, and developed from the elucidation of the chemical basis of specificity described above, and from the use of quantitative methods in the study of the reaction.

The antigen-antibody reaction may be differentiated into two stages, a primary stage concerned with the actual union which occurs very rapidly, and a secondary stage which evolves more slowly as a consequence of that union. Here the primary stage will be considered, and the various manifestations of the secondary stage in the following chapter as the

serological reactions. The secondary stage may be of such a nature that it is somewhat removed from the primary stage, but in the specific precipitation of antigen and antibody in the precipitin reaction, the precipitation is a direct extension of the primary reaction in that the antigen-antibody complex forms aggregates which appear as a visible precipitate. This reaction has been used almost exclusively in study of the antigen-antibody reaction, and for present purposes it is necessary to anticipate this serological reaction here.

Specificity. The nature of the specificity of the union between antigen and antibody and its surface, or adsorptive, character, is indicated by the nature of antigenic, and by inference antibody, specificity as described above. It is dependent upon the juxtaposition and orientation of the haptenic or other determinant of specificity of the antigen with the reaction site on the immune globulin molecule. The antigenic determinant is visualized, at least in the case of artificial conjugated antigens, as projecting from the surface of the protein moiety, and it is believed by some that the corresponding reaction site on the homologous antibody may be in part a crypt in the surface of the globulin molecule, *e.g.*, the shape of the haptene structure is important as well as its constituent atoms. The specificity of the union, then, involves orientation in at least two planes, and possibly also in three; and if the latter holds true, the specificity of the union is analogous to that operative in the formation of mixed crystals.

Intermolecular Forces. When the reaction sites of antigen and antibody have been brought into juxtaposition, they are specifically adsorbed to one another and held together by secondary valencies. These are not covalent bonds, but include electrostatic attraction between oppositely charged groups, dipolar interaction between polar but undissociated groups, hydrogen bonds, and van der Waals forces. None of these are strong bonds, and the last is the weakest, but when the stereochemical configurations of the bound molecule and receptor site are in juxtaposition, they are operative and form a firm, but dissociable, union. Of these, the coulomb forces between oppositely charged groups are possibly the most important as indicated by the marked effect of polar groups on specificity and the relatively high heat of the primary antigen-antibody reaction.

Valence. The valence of antigen or antibody refers to a reaction site, the determinant or haptenic group of the former and the receptor site of the latter. It has been implied earlier that antigen is multivalent in that a number of haptenic groups, such as arsenilic acid, must be attached to the protein moiety to alter its antigenic specificity. The number that are so attached to a pure protein of known molecular weight may be determined by analysis for, for instance, arsenic in the case of arsenilic acid conjugates. The number of such groups that are functional in the union with antibody may be shown by analysis of the antigen-antibody complex to be a function of the size of the antigen molecule. For example, the functional valence of ovalbumin having a molecular weight of about 40,000 is 5 or 6, *i.e.*, this number of molecules of immune globulin may be attached to it when antigen is in excess, that of diphtheria toxin of molecular weight of about 70,000 is 8, and that of the high molecular weight, 650,000, thyroglobulin is about 40. These are minimal values and represent the number of reaction sites that can unite with antibody simultaneously for antibody cannot react with sites too close together because of steric hindrance.

While complete antigens are multivalent, simple haptenes such as arsenilic acid are monovalent, and complex haptenes are multivalent. This is the basis for subdivision of haptenes into two groups as noted earlier. Monovalent haptenes will react with antibody, but complexes are not formed (see below), and the union is demonstrable indirectly by showing that the reacted antibody will no longer react with multivalent antigen. Haptenes may be made divalent by combining, for example, two arsenilic acid

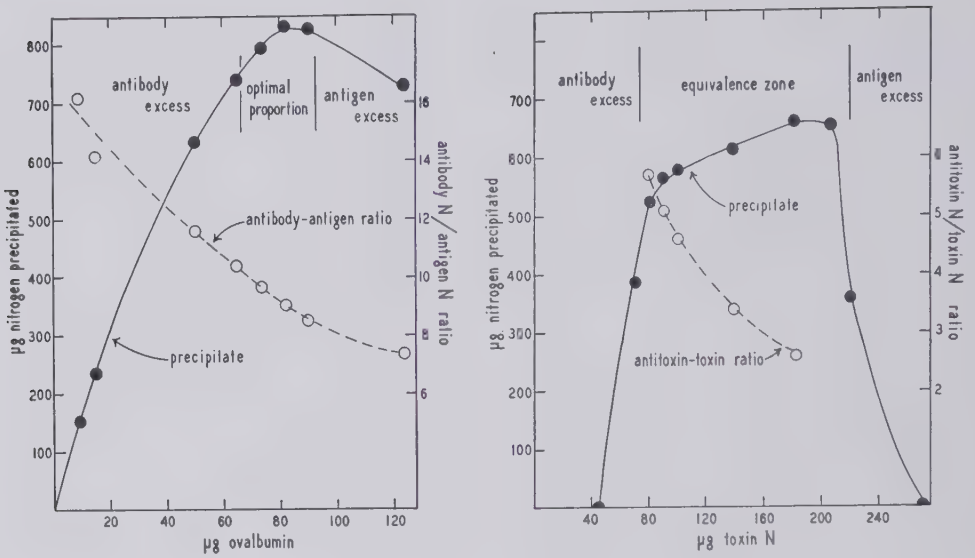


Figure 68. The two types of precipitin systems. *Left*, the "rabbit" type in which the complex is not soluble in the zone of antibody excess; precipitate produced by adding varying amounts of ovalbumin to 1 ml. of rabbit anti-ovalbumin serum. (Data of Heidelberger and Kendall.) *Right*, the "horse" type in which the complex is soluble in excess of either antibody or antigen; total nitrogen precipitated from 1 ml. of diphtheria horse antitoxin by varying amounts of toxin. (Data of Pappenheimer and Robinson.)

groups with some other compound, such as a dye, and such divalent haptenes give antigen-antibody aggregates. Most of the naturally occurring haptenes, such as polysaccharides, are multivalent and react with antibody to form aggregates even though they may not stimulate antibody formation.

It has been pointed out earlier that antibody may be monovalent or divalent, and the former is incomplete, inhibiting, or blocking antibody. In point of fact, there is no definitive evidence that literally monovalent antibody exists; more precisely, some kinds of antibody behave as if they were monovalent and inhibit or block reaction of antigen with divalent antibody. In the case of monovalent haptenes, valency is predetermined, but this is not true of incomplete antibody. It is possible, for example, that in some instances antibody may in fact be divalent but that the reactive sites are too close together to permit reaction with more than one molecule of antigen at the same time.

The existence of divalent antibody is, on the other hand, firmly established by extensive and precise studies showing that, in antigen excess, each antibody molecule combines with two molecules of antigen. The two antibody valencies are apparently always of the same specificity in a single molecule, *i.e.*, in the immune response to two or more antigens given simultaneously a single antibody molecule does not have one valence specific for one antigen, and the other specific for another.

Complex Formation. The initial primary union of antigen and antibody occurs very rapidly and is usually complete within a few minutes, even at low temperatures. The aggregates which appear as a visible precipitate are formed over a longer period, as much as several hours. The aggregate material apparently consists of antibody molecules fairly tightly packed around antigen molecules, with some of the antibody molecules forming bridges between these units to give chains and a loose network of the complex. When either antigen or antibody is monovalent, or behaves as if it were, the available valences are satisfied, bridges, and therefore aggregates, cannot be formed, and there is no precipitate.

Composition of the complex. Whether or not visible aggregates of antigen and antibody occur, their relative content of antigen and antibody is determined by the proportions in which antigen and antibody are mixed. In this respect, three zones are to be distinguished, *viz.;* the zone of antibody excess in which the proportion of antibody to antigen is large, and excess antibody is uncombined and demonstrable in the supernatant; the zone of optimal proportions, or equivalence zone, in which there is no uncombined antigen or antibody; and the zone of antigen excess in which antigen is present uncombined.

With respect to the formation of visible precipitate in the zones of antibody excess and antigen excess, two kinds of systems are differentiable. In one of these, the horse or H type, so-called because it is characteristic of the horse antitoxin-toxin system, precipitation is inhibited in an excess of either reagent. In the other, characteristic of most antigen-antibody systems and called the rabbit or R type because antiserums prepared in the rabbit are usually used, precipitation is not inhibited in the zone of antibody excess, and in this region all of the antigen is precipitated so that the amount of precipitate formed is determined by the amount of antigen added. These two types of precipitating systems are illustrated in the accompanying figure.

The failure to form precipitate, or other manifestation of the antigen-antibody reaction in the zone of antibody excess is called the prozone phenomenon. In the case of the toxin-antitoxin system, this is due to the formation of antigen-antibody complexes which do not aggregate. In practice, however, the prozone phenomenon occurs in all kinds of antigen-antibody reactions and even in *in vivo* protection tests (Schwellenwert). So it need not be due to the solubility of the complex in excess antibody. For example, there may be simply insufficient antigen present to give a precipitate. Or, when adequate amounts of antigen are present, as in bacterial agglutination, the particulate antigen may be rapidly saturated by antibody, leaving no functional valences available for the formation of antibody bridges, *i.e.*, the particles are soluble complexes. The inhibition of precipitation in antigen excess is theoretically due to the formation of soluble complexes, and this may be regarded simply as a

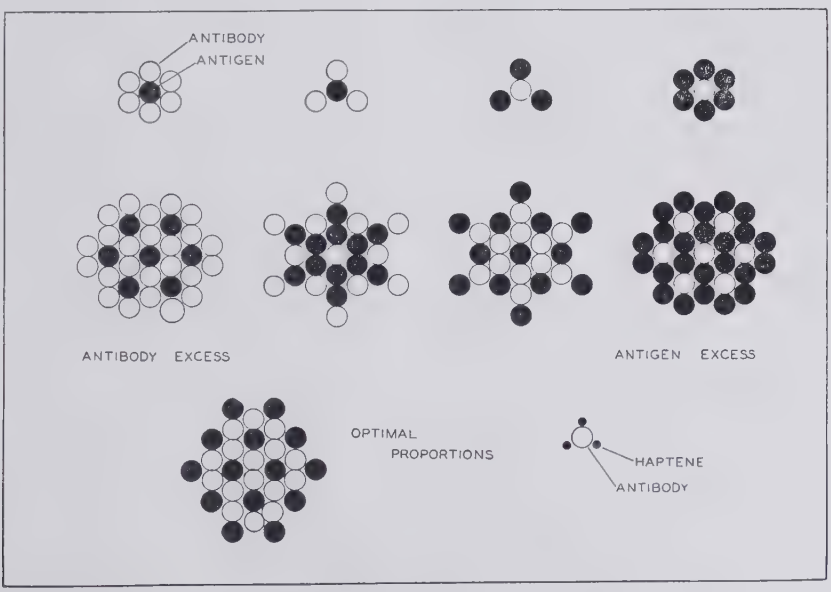


Figure 69. Diagrammatic representation of possible arrangements of antigen and antibody molecules in the complex. (Modified from Marrack.)

consequence of the presence of too small amounts of antibody to aggregate the antigen present.

The bulk of the precipitate formed with soluble antigen is antibody. Measured by weight, the ratio of antibody to antigen may be quite small when the antigen molecule is very large, but with small antigen molecules it may be as much as 100, a consequence of surface-volume relationship in the antigen molecule. The molecular ratio of antibody to antigen may be determined in closely defined systems; with bovine serum albumin antigen and rabbit antibody, for example, the molecular ratio varies from about 6 in the zone of antibody excess to approximately 3 in the zone of antigen excess.

The optimal proportion or equivalence zone is, as indicated above, the antigen-antibody ratio at which neither antigen nor antibody is present in uncombined form. In practice this is readily determined by the precipitin ring test (Chap. 14) applied to the supernatant. This usually, though not invariably, coincides approximately with the antigen-antibody ratio, giving a maximum amount of precipitate, and that at which precipitate first appears. The last criterion is the one usually used in toxin-antitoxin flocculation. The titration may be carried out by using a constant amount of antibody mixed with varying amounts of antigen or by keeping the antigen constant and varying the antibody concentration. The former, designated the α-type procedure by British workers, is usually used, but the latter, the β-type procedure, is used for toxin-antitoxin titration. The two may give slightly different results, designated as the α-type optimum and the β-type optimum respectively, but usually a different β-type optimum is poorly defined or does not occur.

The ratio of antibody to antigen in the zone of optimal proportions is a constant for any given system, *i.e.*, the ratio is the same when the concentrations are varied, and therefore constitutes one of the most precise measures of antibody content of an antiserum. It occurs over a narrow range when a single antigenic specificity is concerned, and the antigen molecules are uniform, but takes the form of a plateau when more than one antigenic specificity is concerned, or when the antigen molecules, such as those of pneumococcal polysaccharide, vary in size and number of combining sites.

Mathematical Analysis. A number of theories of the interaction of antigen and antibody have been proposed, and the modern theories date from Bordet's postulation of the reaction as an adsorption phenomenon. One of the first of these was that of Heidelberger[30] who assumed that the reaction was similar to a chemical one between substances multivalent with respect to one another and behaved according to mass action to give a series of bimolecular reactions. Using the conventional symbols Ab = antibody and Ag = antigen:

$$\begin{aligned} \mathrm{Ag} + \mathrm{Ab} &\rightharpoonup \mathrm{Ag.Ab} \\ \mathrm{Ag.Ab} + \mathrm{Ag} &\rightharpoonup \mathrm{AgAb.AG} \\ \mathrm{AgAb} + \mathrm{AgAb} &\rightharpoonup \mathrm{AgAb.AgAb} \end{aligned}$$

and so on in the region of excess antibody, with the formation of visible aggregates having the general formula $(Ag_nAb_m)_x$. This leads to a general description of the precipitation reaction in the zone of excess antibody:

$$\mathrm{AbN} = 2\mathrm{Rx} - \frac{\mathrm{R}^2}{\mathrm{AgN}}\,\mathrm{x}^2$$

where R = AbN/AgN and x = antigen precipitated. Since it is assumed that the reactions are irreversible, this holds only in the zone of antibody excess.

Hershey's[32, 33] more intensive mathematical treatment is based upon the assumption the antigen-antibody union occurs as a series of reversible reactions which are unaffected by aggregation and may be represented as

$$\begin{aligned} \mathrm{Ag} + \mathrm{Ab} &\rightleftharpoons \mathrm{AgAb} \\ \mathrm{AgAb} + \mathrm{Ab} &\rightleftharpoons \mathrm{Ag(Ab)_2} \\ &\cdots\cdots\cdots\cdots \\ \mathrm{Ag_{n+1}} + \mathrm{Ab} &\rightleftharpoons \mathrm{Ag(Ab)_n} \end{aligned}$$

On analysis, this approach gives the Langmuir absorption equation

$$\mathrm{R} = \frac{\mathrm{ab[Ag]}}{1 + \mathrm{b[Ag]}}$$

in which R = the ratio of antigen in the precipitate [Ag], to the concentration of antibody in the supernatant, and *a* and *b* are constants. Equations of this type fit the linear relation between antigen-antibody ratios in the precipitate to the concentration of antibody in the region of antibody excess, and to the concentration of antigen present in the supernatant in the region of antigen excess.

Using a treatment identical with that of the dissociation of a polybasic acid, and assuming that antibody behaves as if it were monovalent, Teorell[77, 78] arrived at the relationship

$$[Ab] = Ab - [Ag](p_1 + p_2 + \ldots)$$

when [Ab] and [Ag] represent the concentrations of free antibody and free antigen respectively in the supernatant, Ab the total amount of antibody present, and p_1, p_2, etc., represent the values $[Ab]/k_n$, $[Ab]/k_nk_{n-1}$, etc., in which k_n, k_{n-1}, etc., are the several equilibrium constants. This treatment allows a fairly accurate prediction of the course of the precipitation reaction over its entire range when reasonable assumptions of solubilities and valence are made.

The most comprehensive treatment is that of Goldberg[26] which is stochastic in taking into account the known heterogeneity of antibody present in immune serum by calculating the most probable distribution of molecular species by the methods used to study the distribution of molecular size of branched chain polymers. It assumes both divalent and monovalent antibody and multivalent antigen which react to produce branched chains until the system reaches a point at which it consists largely of relatively few large aggregates, and precipitation occurs, but no arbitrary parameters are introduced for the purpose of curve fitting. On this basis m_{ijk}, the number of aggregates composed of *i* divalent antibody molecules, *j* monovalent antibody molecules, and *k* multivalent antigen molecules, is given as

$$m_{ijk} = fG \frac{(fk - k)!}{(fk - 2k + 2 - q - j)!\, k!\, q!\, j!} r^{k-1} p^{k+i+1} \times p^{k+i+j-1}(1 - p)^{fk-k-i-j+1} (1 - \rho\, pr)^{i-k+1} (1 - \rho)$$

when

$$i = k - 1 + q \text{ and}$$
$$0 \leq q + j \leq fk - 2k + 2$$

and G = the number of molecules of antigen in the system, *f* = the valence of each antigen molecule, *q* = the number of free antibody valences in an aggregate, *p* = the proportion of antibody valences in the system that have reacted, ρ = the proportion of antibody valences on divalent antibody molecules, A = the number of divalent antibody molecules in the system, $r = fG/2A$, and $s = fG/D$ where D = the number of monovalent antibody molecules.

Provided that the initial composition of the system, the valence of the antigen, and the extent of the reaction are known, this expression allows the calculation of the number of every kind of aggregate in the system, and the point at which very large aggregates are formed, and precipitation occurs.

The critical condition at which the complex takes the form of very large aggregates to give precipitation may be calculated from a simpler formulation derived from the above, *viz.*:

$$\gamma p^2_c = \frac{1}{V - 1}$$

when γ = the ratio of antigen valence to antibody valence, p_c = the critical fraction of antigen valences bound, and V = the valence of the antigen. It may be calculated that when the valence of antigen is 2, the formation of large aggregates occurs only in a mixture in which there is an equal number of antigen and antibody valences. With higher antigen valence, the range over which precipitation can occur becomes broad; for example, with an antigen valence of 6 precipitation can occur over a twenty-five-fold range in the ratio of antigen valence to antibody valence, and with an antigen valence of 100, the range is 10,000-fold.

The occurrence of precipitation is also affected by the dissociation constant for,

when the concentration of antibody is low relative to its dissociation constant, a significant portion of antigen and antibody valences are uncombined. For example, with an antigen valence of 6, precipitation cannot occur when the antibody concentration is equal to or less than the dissociation constant. It follows also that the critical condition for the formation of very large aggregates may occur in lower concentrations of antibody when the valence of antigen is large. This is consistent with the general observation that the larger the antigen, *e.g.*, bacteria, erythrocytes, etc., the less antibody is required and the higher the apparent antibody titer.

As pointed out earlier, the precipitin reaction may be regarded as a logical extension of the initial antigen-antibody union. It is perhaps incidental, as will appear, that toxin is neutralized by antitoxin, but the cytotoxic effects of antibody, or complement mediated by antibody, when the antigen is cellular would appear to be set somewhat apart from considerations such as these.

REFERENCES

1. Baxter, H. C., and Goodman, J. H.: Nephrotoxic serum nephritis in rats. II Preparation and characterization of a soluble protective factor produced by trypsin digestion of rat tissue homogenates. J. Exptl. Med., 1956, **104:**487–499.
2. Beiser, S. M., Erlanger, B. F., Agate, F. J., Jr., and Lieberman, S.: Antigenicity of steroid-protein conjugates. Science, 1959, **129:**564–565.
3. Billingham, R. E.: Reactions of grafts against their hosts. Science, 1959, **130:**947–953.
4. Boyd, W. C.: Fundamentals of Immunology. Interscience, New York. 1956.
5. Boyd, W. C.: Genetics and Races of Man. Boston University Press, 1958.
6. Boyd, W. C.: Introduction to Immunological Specificity. Interscience, New York. 1962.
7. Boyden, S. V.: Antibody production. Nature, 1960, **185:**724–727.
8. Burger, M.: Bacterial Polysaccharides. Thomas, Springfield, Illinois. 1950.
9. Burnet, F. M.: Enzyme, Antigen and Virus. A Study of Macromolecular Pattern in Action. Cambridge University Press, 1956.
10. Burnet, F. M.: Auto-immune disease. I. Modern immunological concepts. Brit. Med. J., 1959, **ii:**645–650.
11. Burnet, F. M.: Auto-immune disease. II. Pathology of the immune response. Brit. Med. J., 1959, **ii:**720–725.
12. Burnet, F. M.: The Clonal Selection Theory of Acquired Immunity. Vanderbilt University Press, 1959.
13. Burnet, F. M.: Theories of immunity. Perspect. Biol. Med., 1960, **3:**447–458.
14. Burnet, F. M.: The new approach to immunology. New England J. Med., 1961, **264:**24–34.
15. Burnet, F. M.: Immunological recognition of self. Science, 1961, **133:**307–311.
16. Burnet, F. M., and Fenner, F.: The Production of Antibodies. Macmillan, New York. 1950.
17. Bussard, A. D.: Biosynthesis of antibodies, facts and theories. Ann. Rev. Microbiol., 1959, **13:** 279–296.
18. Carpenter, P. L.: Immunology and Serology. W. B. Saunders Company, Philadelphia. 1956.
19. Chase, M. W.: Immunologic tolerance. Ann. Rev. Microbiol., 1959, **13:**349–376.
20. Cole, L. R., and Favour, C. B.: Correlations between plasma protein fractions, antibody titers, and passive transfer of delayed and immediate cutaneous reactivity to tuberculin, PPD and tuberculopolysaccharides. J. Exptl. Med., 1955, **101:**391–420.
21. Cushing, J. E., and Campbell, D. H.: Principles of Immunology. McGraw-Hill, New York. 1957.
22. Dujarric de la Rivière, R., and Eyquem, A.: Les Groupes sanguins chez les Animaux. Editions Medicale Flammarion, Paris. 1953.
23. Freund, J.: Some aspects of active immunization. Ann. Rev. Microbiol., 1947, **1:**291–306.
24. Furth, J., and Kabat, E. A.: Immunological specificity of material sedimentable at high speed present in normal and tumor tissues. J. Exptl. Med., 1941, **74:**247–256.
25. Gitlin, D., Gross, P. A. M., and Janeway, C. A.: The gamma globulins and their clinical significance. II. Hypogammaglobulinemia. New England J. Med., 1959, **260:**72–76.
26. Goldberg, R. J.: A theory of antibody-antigen reactions. I. Theory for reactions of multivalent antigen and bivalent and univalent antibody. J. Amer. Chem. Soc., 1952, **74:**5715–5725.
27. Gorer, P. A.: The antigenic structure of tumors. Adv. Immunol., 1961, **1:**345–394.
28. Hašek, M., Lengerová, A., and Hraba, T.: Transplantation immunity and tolerance. Adv. Immunol., 1961, **1:**1–66.
29. Hauschka, T. S., and Amos, D. B.: Cytogenetic aspects of compatibility. Ann. N. Y. Acad. Sci., 1957, **69:**561–579.
30. Heidelberger, M.: Quantitative absolute methods in the study of antigen-antibody reactions. Bact. Rev., 1939, **3:**49–95.
31. Henle, W., Chambers, L. A., and Groupé, V.: The serological specificity of particulate components derived from various normal mammalian organs. J. Exptl. Med., 1941, **74:**495–510.
32. Hershey, A. D.: A descriptive theory of specific precipitation. I. The theory. II. Quantitative applications. III. The individuality of antibody. J. Immunol., 1941, **42:**455–530.
33. Hershey, A. D.: Specific precipitation. IV. Quantitative application of the stricted theory. J. Immunol., 1942, **45:**39–50.
34. Hummel, K.: Die inkompletten Antikörper in der Immunobiologie. Gustav Fischer, Stuttgart. 1955.

35. Irwin, M. R.: Immunogenetics. Adv. Genetics, 1947, **1:**133–159.
36. Jerne, N. K.: Immunological speculations. Ann. Rev. Microbiol., 1960, **14:**341–358.
37. Kabat, E. A.: Blood Group Substances: Their Chemistry and Immunochemistry. Academic Press, New York. 1956.
38. Kabat, E. A.: Kabat and Mayer's Experimental Immunochemistry. Thomas, Springfield, Illinois. 3rd ed., 1961.
39. Kunkel, H. G., Fudenberg, H., and Ovary, Z.: High molecular weight antibodies. Ann. N. Y. Acad. Sci., 1960, **86:**966–973.
40. Landsteiner, K.: The Specificity of Serological Reactions. Harvard University Press, 1945.
41. Lederberg, J.: Genes and antibodies. Science, 1959, **129:**1649–1653.
42. Lennox, E. S.: Antigenic analysis of cell structure. In Gunsalus, I. C., and Stanier, R. Y., (eds.): The Bacteria, vol. 1, pp. 415–442. Academic Press, New York. 1960.
43. Lippman, R. W., Marti, H. U., and Campbell, D. H.: Nephrotoxic globulin nephritis, course after single intravenous injection. Arch. Path., 1952, **53:**1–14.
44. Marrack, J.: The Chemistry of Antigens and Antibodies. Medical Research Council (Great Britain) Spec. Rept. No. 230. 1939.
45. Marrack, J.: The structure of antigen-antibody aggregates and complement fixation. Ann. Rev. Microbiol., 1955, **9:**369–386.
46. Maurer, P. H.: The antigenicity of polyvinyl pyrrolidone. J. Immunol., 1956, **77:**105–110.
47. Medawar, P. B.: Immunological tolerance. Science, 1961, **133:**303–306.
48. Menolasino, N. J., Goldfin, M., and Hoffman, A.: Thermal differentiation between H and O agglutinins. I. Pseudomonas and Alcaligenes. J. Bact., 1955, **69:**270–274.
49. Morales, M. F., Botts, J. and Hill, T. L.: Equilibrium equations for a model of antigen-antibody combination. J. Amer. Chem. Soc., 1948, **70:**2339–2341.
50. Mourant, A. E.: The Distribution of Human Blood Groups. Thomas, Springfield, Illinois. 1954.
51. Mourant, A. E.: The ABO Blood Groups. Blackwell, London. 1958.
52. Nuttall, G. H. F.: Blood Immunity and Blood Relationship. Cambridge University Press, 1904.
53. Pauling, L.: A theory of the structure and process of formation of antibodies. J. Amer. Chem. Soc., 1940, **62:**2643–2657.
54. Pauling, L., and Campbell, D. H.: The manufacture of antibodies in vitro. J. Exptl. Med., 1942, **76:**211–220.
55. Pauling, L., Campbell, D. H., and Pressman, D.: The nature of the forces betweeen antigen and antibody and of the precipitation reaction. Physiol. Rev., 1943, **23:**203–219.
56. Pierce, A. E.: Specific antibodies at mucous surfaces. Vet. Rev. Annot., 1959, **5:**17–36.
57. Pressman, D., and Sherman, B.: The zone of localization of antibodies. XII. Immunological specificities and cross reactions in the vascular beds of liver, kidney and lung. J. Immunol., 1951, **67:**21–33.
58 Race, R. R., and Sanger, R.: Blood Groups in Man. Thomas, Springfield, Illinois. 1958.
59. Raffel, S.: Immunity. Appleton-Century-Crofts, New York. 2nd ed., 1961.
60. Schultz, J.: Antigens and antibodies as cell phenotypes. Science, 1959, **129:**937–943.
61. Sevag, M. G.: Immuno-catalysis. Thomas, Springfield, Illinois. 2nd ed., 1951.
62. Singer, S. J., Fothergill, J. E., and Shainoff, J. R.: A general method for the isolation of antibodies. J. Amer. Chem. Soc., 1960, **82:**565–571.
63. Smith, R. T.: Immunological tolerance of nonliving antigens. Adv. Immunol., 1961, **1:**67–131.
64. Snell, G. D.: The homograft reaction. Ann. Rev. Microbiol., 1957, **11:**439–458.
65. Snell, G. D.: The genetics of transplantation. Ann. N. Y. Acad. Sci., 1957, **69:**555–560.
66. Stack, M. V.: The action of enzymes on antibodies. Bull. Hyg., 1947, **22:**733–741.
67. Symposium: The Nature and Significance of the Antibody Response. Columbia University Press, 1953.
68. Symposium: A discussion of immunological tolerance. Proc. Roy. Soc., Ser. B, 1956, **146:** 1–92.
69. Symposium: Antibodies. Their production and mechanism of action. J. Cell Comp. Physiol., 1957, **50:** Suppl. 1.
70. Symposium: Fourth tissue homotransplantation conference. Ann. N. Y. Acad. Sci., 1960, **87:** 1–607.
71. Symposium: Connective tissue and diseases of connective tissue. Ann. N. Y. Acad. Sci., 1960, **86:**875–1132.
72. Symposium: Fifth tissue homotransplantation conference. Ann. N. Y. Acad. Sci., 1962, **99:** 335–942.
73. Symposium: Blood groups in infrahuman species. Ann. N. Y. Acad. Sci., 1962, **97:**3–328.
74. Taliaferro, W. H.: Synthesis and degradation of antibody. J. Cell. Comp. Physiol., 1957, **50:** Suppl. 1, pp. 1–26.
75. Talmage, D. W.: Immunological specificity. Science, 1959, **129:**1643–1648.
76. Tanaka, N., and Leduc, E. H.: A study of the cellular distribution of Forssman antigen in various species. J. Immunol., 1956, **77:**198–212.
77. Teorell, T.: Quantitative aspects of antigen antibody. I. A theory and its corollaries. J. Hyg., 1946, **44:**227–236.
78. Teorell, T.: Quantitative aspects of antigen-antibody. II. Some comparisons between the theory and the experimental results. J. Hyg., 1946, **44:**237–242.
79. Thomas, L., Paterson, P. Y., and Smithwick, B.: Acute disseminated encephalomyelitis following immunization with homologous brain extracts. I. Studies on the role of the circulating antibody in the production of the condition in dogs. J. Exptl. Med., 1950, **92:**133–152.
80. Weil, A. J.: The Wassermann antigen and related "alcohol-soluble" antigens. Bact. Rev., 1941, **5:**293–330.
81. Wiener, A. S.: Advances in Blood Grouping. Grune and Stratton, New York. 1961.
82. Wiener, A. S., and Wexler, I. B.: Heredity of the Blood Groups. Grune and Stratton, New York. 1958.
83. Witebsky, E., Rose, N. R., Paine, J. R., and Egan, R. W.: Thyroid-specific auto-antibodies Ann. N. Y. Acad. Sci., 1957, **69:**669–677.

Chapter 14

IMMUNITY
—The Serological Reactions

THE VARIETY of consequences of the primary union of antigen and antibody make up the so-called second stage of the antigen-antibody reaction. These occur *in vitro* and, with the exception of certain kinds of protection tests (see below), are test tube reactions. Since the antibody is usually that contained in serum, these reactions are called serological reactions, and the general subject matter, serology. Strains of microorganisms differing in their antigenic structure and separated and identified by serological means are, for example, serological types or serotypes.

The serological reactions are extremely useful for a wide variety of purposes including serological identification of microorganisms and the resolution of their antigenic structure, measurement of the immune response, serodiagnosis of infectious disease by testing patients' serum for the presence of antibody to suspected etiological agents, etc.

The nature of the secondary stage of the antigen-antibody reaction is dependent upon the way the test is carried out, as noted earlier. In general, two kinds of effects are observed, the one an aggregation of soluble or particulate antigen in an overtly apparent way, and the other a cytotoxic action when the antigen is a living cell, that may result in death of the cell, changes in its permeability, or actual dissolution of the cell structure, all of which may be observed *in vitro*.

On the basis of the way in which the secondary stage occurs, several "kinds" of antibodies are distinguished. These are:

The *precipitins* or precipitating antibodies which aggregate soluble antigens.
The *agglutinins* which agglutinate or aggregate particulate antigens, including cells in suspension.
The *antitoxins* which specifically neutralize the pharmacological action of microbial toxins, especially exotoxins.
The *lysins* or antibodies which mediate dissolution of part or all of the structure of a cellular antigen by complement.
The *opsonins* which markedly facilitate the ingestion of cellular or other particulate antigens by the phagocytic cells of the body.
The *protective* or *neutralizing* antibodies which neutralize the infectivity of pathogenic microorganisms and protect the animal against infection and disease.

In addition to these, an animal may be made hyperreactive or hypersensitive to antigenic substances by a preliminary inoculation, and the consequences of antigen-antibody union are apparent only in living tissue, *in vivo*, or *in vitro* in certain kinds of preparations. This will be considered elsewhere (Chap. 15), and the various serological reactions here.

PRECIPITINS

The precipitation of soluble antigen in the presence of homologous antibody by

the formation of large aggregates of the antigen-antibody complex has been described earlier (Chap. 13). In this kind of serological reaction the antibody functions as a precipitin, and the antigen which provokes its formation is known as a precipitinogen. The physical state of the antigen used for immunization is not of particular importance; antibacterial serums, for example, will give precipitates when mixed with preparations of the soluble cell substance of the microorganisms, and precipitins may almost always be demonstrated in lytic, antitoxic, opsonic and agglutinating antiserums.

Precipitation does not occur in the absence of electrolyte and does not require the presence of heat-labile substances such as complement.

The precipitin test is carried out with undiluted, or only slightly diluted, serum, but the antigen solution is diluted in series. The reagents may be mixed in the usual way, or the antiserum may be pipetted into very small test tubes and the solutions of diluted antigen carefully layered on top with no mixing. In the first instance a precipitate is formed which settles out like agglutinated bacteria, while in the second a precipitate forms at the interface between serum and antigen solution. The latter test is termed a "ring test." The end point of either test may be taken as the highest antigen dilution with which the serum forms an observable precipitate and the titer of the serum given in these terms. Potent antiserums may have extremely high titers, precipitating antigen in dilutions of 1:100,000 to 1:5,000,000.

The Quantitative Precipitin Reaction.[12] While such precipitin titers are indicative of the amount of antibody present, a more precise measure is the amount of antibody precipitated from a known amount of antiserum at the equivalence zone. The test is usually carried out by adding increasing amounts of antigen to a constant amount of antiserum. The end point is indicated approximately as that proportion of antigen and antibody which gives the maximum amount of precipitate, and precipitation usually appears first at this point. The equivalence zone, it will be recalled, is that proportion of antigen and antibody such that neither is in excess, *i.e.*, present in the supernatant, and is demonstrated by application of the precipitin ring test to the supernatant. Precipitate formed at the equivalence zone may be removed by centrifugation and analyzed for nitrogen by the usual micromethods to give, by difference, the total antibody nitrogen present. In precise quantitative work the co-precipitation of other nitrogenous substances must be taken into consideration. The largest contribution of this kind is complement (see below), and for accurate results the antiserum examined must be decomplemented by treatment with some heterologous antigen-antibody system.

Precipitin Reactions in Gels.[3, 50] When antiserum is incorporated in a gel, such as gelatin or agar, and antibody allowed to diffuse into the gel, or when antigen and antibody diffuse toward one another in a gel, the precipitin reaction occurs as a band of precipitate. This phenomenon is known as immunodiffusion. The band is sharp when the proportion of reactants approximates that of the equivalence zone, but commonly this is not true, and the leading edge is sharp while the trailing edge is more diffuse. Since individual antigens form individual bands of precipitate with their homologous antibodies, the gel diffusion method allows the demonstration of antigenic heterogeneity because a mixture of antigens gives multiple bands of precipitation. The number of components so observed is a minimum for there is no assurance that other components are not present but in too small amount to produce visible bands, and what appear to be single bands may sometimes be differentiated or resolved into more than one band.

There are a number of variations in the gel diffusion technique. The single diffusion method of Oudin makes use of antiserum-containing agar or gelatin in a small bore tube which is overlaid with a solution of antigen. On standing for some days, the antigen diffuses into the anti-

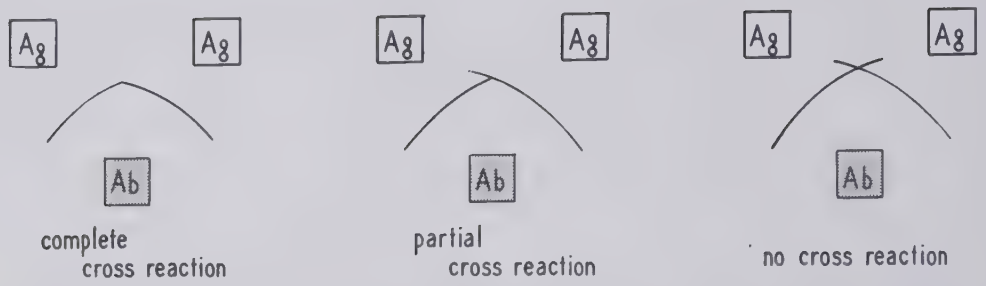


Figure 70. Diagrammatic representation of the Ouchterlony double diffusion technique with single bands of antigen-antibody precipitate. *Left,* the juncture of the bands indicates identity of the antigens; *center,* a partial cross-reaction; and *right,* the independent behavior of the bands indicates no relation between the antigens. In all cases the antiserum contains antibody to both antigens.

body-containing gel with the development of bands of precipitation. In a single antigen-antibody system only one band is formed, while multiple systems give a group of bands corresponding to the number of systems present in appropriate amounts and proportions.

A double diffusion technique was developed by Ouchterlony in which antigen and antibody are placed in wells in an agar gel and allowed to diffuse toward one another to form a band of precipitate. Elek has used a similar method in which strips of filter paper soaked in antigen solution and in antiserum are placed on the surface of an agar gel. In the double diffusion technique multiple wells may be used, *e.g.,* two antigen solutions may diffuse simultaneously toward the same antiserum. The bands of precipitate forming between each of the antigens and the antiserum fuse at their juncture when the two antigens are the same but cross independently when the two antigens are different.[19] At the same time, multiple precipitates may be formed by a single antigen-antibody system, usually when the immunodiffusion is carried out with strongly unbalanced reactant ratios, or when the process is subjected to sudden changes in temperature.

Assay of antigen, or antibody, mixtures by precipitation in gels is based not only on the formation of successive bands of precipitate, but also on the rate at which they diffuse through the gel. The relation between the logarithm of the antigen concentration and square of the migration rate is linear under conditions of free diffusion, and superimposed bands may sometimes be resolved by adjusting the concentrations of the reactants. Oudin, for example, showed that the band of precipitate advances in the single diffusion method according to

$$h = k\sqrt[2]{t}$$

in which h = the distance traversed by the leading edge of the band, t = time, and k is a constant. Since k varies with the concentration of antigen and antibody, it is an index of the concentration of either reactant when the other is known. The viscosity of the gel, the presence of contaminants in the reactants, etc., affect k, and in general the quantitative approach to gel diffusion has not as yet given precise results.

The method has, however, been extremely useful qualitatively. For example, it has been possible to demonstrate by this means the presence of more than one antigenic component in highly purified diphtheria toxin otherwise apparently homogeneous. It also has practical application in this connection in the "*in vitro* virulence test" for toxigenic diphtheria bacilli; the bacteria are grown as a streak on an agar medium near to a strip of filter paper soaked in antitoxin, and a band of precipitate forms between the two when the bacterial strain is toxigenic.

Immunoelectrophoresis.[20, 26, 27] The technique of immunodiffusion may be combined with electrophoretic separation of one of the reactants, commonly the antigen. This is carried out by preparing the gel, commonly agar, in a thin

strip on the surface of glass, applying the antigen in a well in the gel, and separating the components of differing mobility, the gel serving as a supporting medium. The separated material is then reacted with antiserum placed in a trench extending the length of the separation. Bands of precipitate occur about the separated antigenic components which may be stained, photographed, etc. This kind of immunoelectrophoretic analysis is usually carried out on a micro scale, *i.e.*, in thin layers of gel on a glass microscope slide.

Precipitin Reactions with Haptenes. The precipitin reaction occurs not only between antibody and complete antigen but also with some partial antigens or haptenes. An antipneumococcus serum, for example, will react not only with a solution of the pneumococcus cell substance but also with pure capsular polysaccharide. When the haptene is of high molecular weight, as in the case of the polysaccharides, a visible precipitate is formed, but when it is a simple compound such as an organic acid or monosaccharide no observable precipitate appears. The reaction between antibody and such haptenes may, however, be shown by the fact that antiserums treated with haptene will no longer precipitate the complete antigen; the antibody has already reacted with the partial antigen.

Cross-Reactions. The precipitin reaction, like the other immunological reactions, is highly specific, but cross-reactions are observed between similar antigens. With naturally occurring antigens, antigenic relationships tend to parallel phylogenetic relationships, as among the serum proteins of related animal species described elsewhere (Chap. 13). Among artificial conjugated antigens, cross-reactions are associated with similarities in the structures of the attached haptenes. In precipitin and other immunological cross-reactions between naturally occurring antigens, it has not been possible to differentiate unequivocally between those occurring as a consequence of similar determinant structures and those that occur because of the presence of the same antigenic specificity in two otherwise differentiable antigenic mosaics.

In any case, the specificity of the precipitin reaction is such that it differentiates sharply between unrelated soluble antigens and is the serological reaction usually used for the identification of blood stains, etc., for forensic purposes.

AGGLUTININS

If the blood or serum of an animal previously immunized against a bacterium is mixed with a suspension of the microorganisms, the latter become immobilized and in a short time aggregate to form large clumps of cells. In the slide agglutination test, commonly used for rapid identification purposes, the antigen is used as a milky suspension and the antiserum undiluted or only slightly diluted; the suspended bacteria aggregate in the presence of homologous antibody within a minute or two to give a curdled appearance to the suspension. In the test tube these clumps settle out, and the turbid bacterial suspension is cleared with the formation of a precipitate-like mass of clumped cells in the bottom of the tube. This phenomenon is termed *agglutination,* and the bacterial cells are said to be *agglutinated.* The bacteria are not killed by agglutination and will, in fact, grow in immune serum although with altered morphology and the formation of long chains of bacillary forms. Living bacteria need not be used for the agglutination reaction, for dead bacteria are agglutinated as readily as the viable forms.

Although many species of bacteria may be clumped by "normal" serums in low (1:5 to 1:10) dilutions, the capacity of a serum to agglutinate bacteria is greatly enhanced by immunization, high-titered antiserums may be prepared which will bring about agglutination in dilutions of 1:20,000 to 1:50,000. The agglutination reaction is, then, an antigen-antibody reaction, and the antibody is designated an *agglutinin.* The antigen

is sometimes termed an *agglutinogen.* The agglutinin does not require the cooperation of complement or other heat-labile substances, and inactivated serums will agglutinate to titer.

Not only bacteria but a variety of free cells, including erythrocytes and others, are agglutinated by normal and immune serums. The incompatibility of human blood groups is a consequence of the presence of hemagglutinins. Hemagglutinins are also formed by some bacteria and may possibly be instrumental in the formation of the thrombi observed in the blood vessels after death from certain of the infectious diseases.

Agglutination is highly specific, an antiserum agglutinating only the homologous antigen, and agglutination does not require complement. The reaction may be observed microscopically by mixing a suspension of bacteria and diluted antiserum on a slide but is most commonly carried out by mixing the two in 0.5 to 1.0 ml. amounts in small test tubes and observing the formation of a precipitate. In the latter instance, varying dilutions of serum (frequently prepared in geometrical progression by mixing with an equal amount of physiological salt solution, *i.e.*, 1:10, 1:20, 1:40, 1:80, etc.) are added to the bacterial suspension and incubated at 37° C. overnight or at 55° C. for two hours. The highest dilution showing observable flocculation is taken as the titer of the serum; a serum showing agglutination in a dilution of 1:10,000 but not in 1:20,000 is said to have an agglutinin titer of 1:10,000. Such titers are variable to some degree, depending upon the density of the bacterial suspension and other factors; light suspensions, for example, showing only a faint turbidity to the eye may give somewhat higher agglutinin titers than heavy suspensions.

Conglutination and Conglutinating Complement Adsorption.[13, 31, 33, 34] It was observed by Bordet and Gay in 1906 that fresh serum of some animals will agglutinate erythrocytes treated with cow serum. The cow serum is the source of two factors: natural antibody for the erythrocytes, and the clumping activity, conglutinin. The reaction was termed conglutination to distinguish it from agglutination which does not require complement. This reaction may be used as an indicator system in the same manner as the sheep cell–hemolysin system of the complement-fixation reaction, the presence or absence of free complement being indicated by conglutination or conglutination inhibition. Used in this way, it is called a conglutinating complement adsorption test (CCAT). It has become of renewed interest in connection with the serodiagnosis of Q fever and viruses of the psittacosis-lymphogranuloma group.

Passive Agglutination.[47] Particles upon which soluble antigen is adsorbed acquire in a passive sense the antigenicity of that antigen and behave as if they were composed of that antigen. They agglutinate in antiserum to the soluble antigen, and the agglutination is said to be passive agglutination. This phenomenon illustrates the essential identity of precipitin and agglutinin, *i.e.*, in the precipitin reaction molecules are aggregated or agglutinated, while in the agglutination reaction particulate material is agglutinated.

Antigen may be absorbed on collodion, latex or similar particles of appropriate size, but red blood cells, usually of the sheep, have been the most commonly used for this purpose, and the reaction is a passive hemagglutination. Soluble antigens are usually adsorbed relatively rapidly, polysaccharides in 10 to 30 minutes, and proteins a little more slowly. The adsorption of protein may be facilitated by a preliminary treatment of the red cell with dilute (1:20,000) tannic acid. A variety of antigens have been used, including polysaccharide haptenes, soluble antigens from microorganisms such as tuberculin, the O and Vi antigens of enteric bacilli, etc., bacterial toxins such as tetanus toxin, and certain viruses such as adeno-viruses.

The original antigenicity of the red cell is not lost when a soluble antigen is adsorbed on its surface. This is a significant factor when sheep erythrocytes and rabbit antiserums are used, for these

cells contain Forssman antigen, and antibody to this antigen is commonly found in serums of animals, such as rabbits, which do not contain the antigen. Consequently, sheep cells are often agglutinated in rabbit serum, and if these reagents are used, the antiserum must be treated with normal sheep cells to remove the Forssman antibody by absorption (see below) before use. Or this artifact may be avoided by using rabbit erythrocytes.

The passive hemagglutination test is often more sensitive than bacterial agglutination owing to the relatively large size of the antigen particle. Antibacterial serums having homologous antibacterial agglutinin titers of 1:5000 to 1:10,000 may agglutinate red cells sensitized with soluble bacterial antigen to titers of 1:50,000 or more.

The antiglobulin reaction.[14] The antiglobulin reaction is a passive agglutination in which the antigenic cell or particle is treated, first with antiserum specific for its surface antigen, and then with antiserum to the serum globulin of the specific antibody. In the first stage of the reaction, an ordinary agglutination or passive agglutination, homologous antibody in the form of immune globulin reacts specifically with its antigen. When the immune globulin is thus specifically adsorbed onto the particle, the particle behaves antigenically as if it were serum globulin. On the addition of antibody to the globulin, antiglobulin, the immune globulin that acted as an antibody in the first instance now acts as an antigen, and the coated particles are agglutinated. While, then, in a technical sense the second reaction is specific, the specificity of the antiglobulin reaction as a serological reaction lies in the specificity of the original antigen and its antibody.

This reaction is particularly useful for the titration of incomplete antibody which will of itself not agglutinate a particulate antigen. It does, however, react with antigen to adsorb onto a particulate antigen, which then becomes susceptible to agglutination with antiglobulin serum. In other words, the incomplete antibody behaves as if it were monovalent, but as an antigen it is multivalent. For example, a bacterial antigen is agglutinated in its homologous antiserum by the complete antibody present. If, however, antibody to the immune globulin is added to the system, the bacterial cells which reacted with incomplete antibody will now agglutinate. Antiglobulin titers are frequently higher than agglutination titers, because the latter titrates only complete antibody, while the former titrates both complete and incomplete antibody. In some instances, notably that of Rh antibody, antibody is largely incomplete and agglutination occurs, for all practical purposes, in the antiglobulin reaction.

Cross-Reactions. Although the agglutination reaction is highly specific, certain cross-reactions between closely related bacterial species are frequently

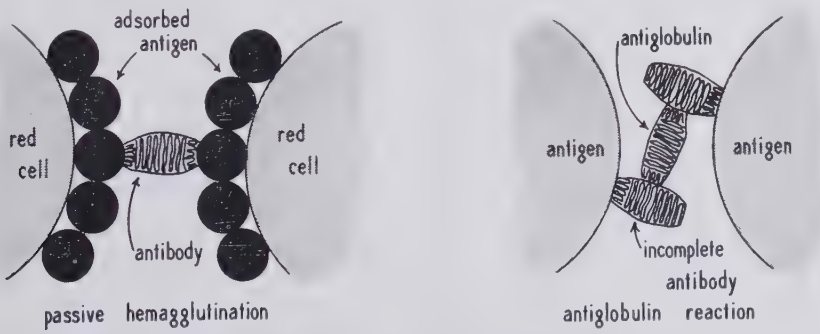


Figure 71. Diagrammatic representation of the passive hemagglutination reaction (*left*) and the antiglobulin reaction (*right*). Note that the antigen-antibody reaction is between antibody and adsorbed antigen in the first instance and between incomplete antibody globulin molecules and their homologous antibody in the second.

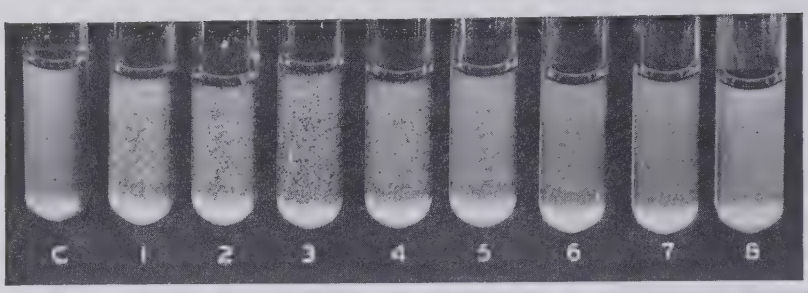


Figure 72. The macroscopic agglutination test—flagellar agglutination of Salmonella. The control tube, *C*, contains bacterial suspension but no antiserum. Successive dilutions in the numbered tubes are 1:100, 1:200, 1:500, 1:1000, 1:2000, 1:5000, 1:10,000, and 1:20,000. Note agglutination in 1:10,000 but not in 1:20,000.

observed. Such reactions are attributable not to a lack of immunological specificity but to the immunological heterogeneity of the bacterial cell. As indicated earlier (Chap. 13), the cell is made up of a variety of antigenic components, an "antigenic mosaic." Clearly, then, if the same component is present in each of two species of bacteria, an antiserum prepared against the one will agglutinate the other but generally to a reduced titer, *i.e.*, only in the lower serum dilutions. This sharing of antigenic components and the resulting cross-reactions are particularly common among the gram-negative enteric bacilli. The agglutinins responsible for such cross-reactions are sometimes referred to as "group agglutinins" and the phenomenon as "group agglutination."

Agglutinin Absorption. The direct demonstration of the antigenic heterogeneity of a bacterium and the consequent multiple antibody content of its homologous antiserum are made possible by agglutinin absorption. If a heavy suspension of bacteria is prepared in diluted (commonly 1:5) antiserum, incubated for two to three hours and centrifuged, the supernatant diluted serum will be found to have lost its ability to agglutinate the bacterium with which it was absorbed; the agglutinins for that microorganism have been taken up by the bacterial cells, leaving other agglutinins intact. In practice it is necessary to absorb two or three times and, since the phenomenon is an adsorption, it is not always possible to remove completely the agglutinins in question.

This preferential absorption makes possible the breakdown of the antigenic mosaic of the bacterial cell into component parts. For example, an antiserum prepared against a bacterium containing antigens *A*, *B*, and *C* will contain antibodies *a*, *b*, and *c*. If such an antiserum is absorbed by a second bacterium which contains antigens *B* and *C*, antibodies *b* and *c* will be removed leaving *a* intact, and the antiserum will still agglutinate its homologous bacterium. Suppose an antiserum is prepared against the second bacterium which will contain antibodies *b* and *c*. If this serum is absorbed by the bacterium containing antigens *A*, *B*, and *C*, it will no longer agglutinate its homologous antigen, for all the antibodies will have been removed. Clearly, then, the complete absorption of a known serum by an unknown organism does not indicate that the unknown organism is necessarily immunologically identical with that with which the known serum was prepared; to prove such immunological identity a "mirror absorption" must be carried out, *i.e.*, each antiserum absorbed with each organism.

The agglutination test and agglutinin absorption, although used to some extent in the diagnosis of infectious disease (as in the Widal test in which typhoid bacilli are mixed with patient's serum), have been found particularly valuable in the study of the relation of bacterial species to one another and in some instances, as in the case of the Salmonella (Chap. 19), complex antigenic formulas have been worked out. The determination of the components of the antigenic

mosaic of a bacterial species is termed *antigenic analysis.*

H and O Agglutination. A number of bacterial species, particularly Salmonella, Proteus, and certain others, may agglutinate in two ways.

Macroscopically the H agglutination gives rise to a loose, flocculent precipitate, and upon microscopic examination it may be observed that the bacterial clumps are loose, the flagella of the microorganisms being entangled with one another. The O agglutination, on the other hand, produces a finely granular precipitate in which the individual bacterial cells are closely packed together. These types of agglutination are a consequence of the presence or absence of the flagellar, heat-labile H antigen. Immunization with the whole bacterial cell containing both flagellar and somatic antigenic components gives rise to antiserums containing both types of antibody, the H generally in high titer while the O antibody is commonly active in dilutions of less than 1:1000.

Spontaneous Agglutination. Some strains of bacteria do not form stable suspensions and are said to be spontaneously agglutinable. This behavior is particularly characteristic of rough variants and, although not an immunological phenomenon, is frequently of practical importance in agglutination studies.

Cold Agglutinins. In some diseases so-called cold agglutinins, which agglutinate human O group erythrocytes at 0° C. but not at 37° C., are present in the serum. They are found in one half or less of cases of primary atypical pneumonia and have limited utility in eliminating certain other infections, such as Q fever, in the diagnosis of this disease. The titer reaches a peak early in convalescence, but the agglutinin has, so far as is known, no immunological status, for it is found in other diseases such as trypanosomiasis and hemolytic anemias, and it may appear following sulfonamide therapy.

The Mechanism of Agglutination. The clumping of bacteria under the influence of immune serum may be taken as evidence per se that a force attracting the cells to one another is operative, at least at times. Similarly, the fact that bacteria are not in a constant state of agglutination is indicative of a force which tends to hold the cells apart from one another. The attractive or cohesive force is probably that of surface tension, *i.e.,* the interfacial tension at the cell surface, and the repulsion that of like electrical charges, for, as pointed out previously, bacteria are negatively charged at pH's compatible with viability. On the basis of such reasoning it would appear that the balance between these opposing forces determines whether the microorganisms will form a stable suspension or whether they will clump together and settle out.

It was early observed that the presence of an electrolyte is essential to agglutination; if both immune serum and bacterial suspension are dialyzed free of salt before mixing, the cells are not agglutinated, but if a trace of salt is added to the mixture, agglutination takes place at once. This behavior, it will be seen, corresponds to that of a mixture of two colloids of opposite charge, such as gum mastic and gelatin, when the one is added in too small an amount to precipitate in the absence of salt. That the repelling effect of electrical charge is an important factor is also indicated by the agglutination of bacteria in the absence of antibody when the pH is lowered to the iso-electric point of the cells, a phenomenon termed *acid agglutination.* It was formerly thought that bacterial species might be sharply differentiated from one another on the basis of the pH of their acid agglutination, but this has not proved to be true.

The agglutination of bacterial cells is, however, a function not only of electrical charge on the cells but also of the cohesive forces tending to draw them together. Studies in which both potential difference (between the cells and the suspending medium) and cohesive force were directly measured have shed considerable light on the mechanism of agglutination. It has been found that electrolytes in low concentration (0.01 N) affect primarily the potential, and in

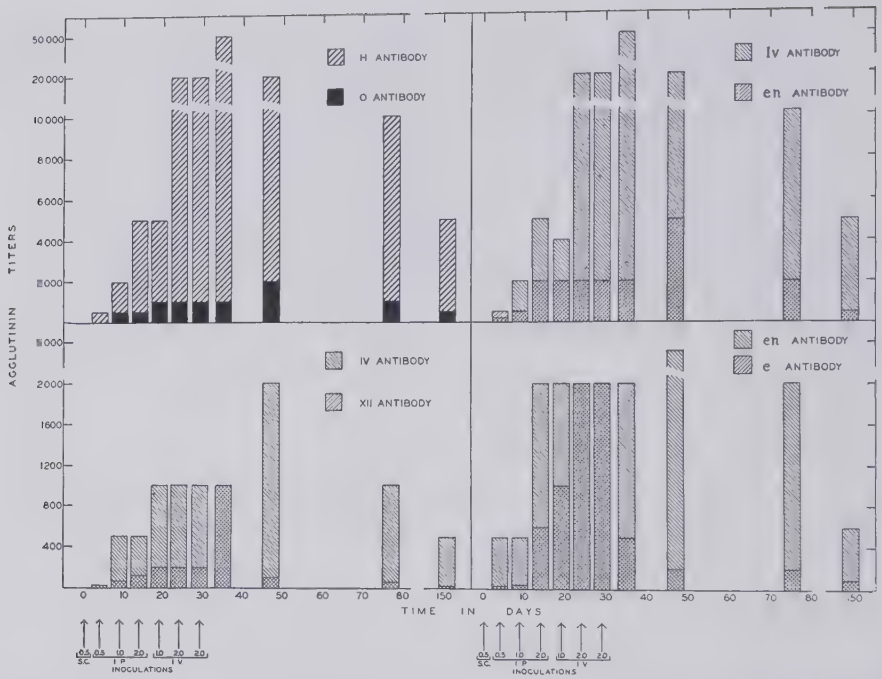


Figure 73. The immune response in the rabbit to the antigenic complex of *Salmonella brandenburg*. *Upper left,* H and O agglutinin titers, showing the usual marked discrepancy between the two. *Lower left,* the differential response to the two components of the O antigen. *Upper and lower right,* the response to the components of the H antigen. Note the independent behavior of the antibodies as indicated by variation in peak titers. All titers determined by direct agglutination of heterologous antigens without agglutinin absorption.

high concentration also decrease the cohesive force. If the cohesive force is not affected, agglutination occurs when the potential is reduced below the critical point of 15 mv. If, however, the cohesive force is decreased, the critical potential is also decreased, and, therefore, in concentrated salt solutions agglutination does not take place even though there is no measurable potential. Immune serum, presumably adsorbed on the surface of the bacterial cells, while reducing the charge somewhat appears to function by preventing the salt from decreasing the cohesive force, and agglutination occurs at the critical point of 15 mv. Furthermore, if the potential difference is reduced by electrolyte to 15 mv. or less in a bacterial suspension, the addition of immune serum raises the reduced cohesive force, and agglutination takes place. Examination of typhoid bacilli by the electron microscope in the presence of immune serum has shown that the flagella become thickened by the deposition of an antibody film approximately 21 Å thick, and the cell walls become more opaque and less definite in outline. The serum-sensitized surfaces appear to be sticky, not only for one another, but also for other particulate matter.

Serological adhesion.[37, 60, 61] This stickiness may be in part at least the basis of the phenomenon of serological adhesion in which nonspecific particulate material tends to adhere to particulate antigen in the presence of antibody and complement. It was first observed *in vivo* as an adhesion of platelets to cholera vibrios, trypanosomes, and several kinds of spirochetes to provide the basis for serodiagnostic tests. It is variously known as the Levaditi phenomenon, the Laveran-Mesnil reaction, the Rieckenberg reaction, and as the Brussin or Beladung reaction in the Russian literature. What is probably the same phenomenon was later called immune adherence in a reaction in which bacteria, especially syphilis spirochetes, ad-

here to red blood cells in the presence of antibody and complement.

The nature of this reaction is obscure. It may be observed *in vitro* as well as *in vivo*; various kinds of particles will function in the reaction, but there are discrepancies as among red cells of varied origin, and adhesion has been found to occur with some bacterial antigens but not with others such as Salmonella. This reaction, using human erythrocytes as the adhering particles, has been developed by Nelson as a diagnostic test for syphilis, the *Treponema pallidum* immune adherence, or TPIA, reaction in which the spirochetes are mixed with complement, antiserum, and red cells, and the adherence of the red cells to the spirochetes is observed microscopically *in vitro*.

Blocking antigens. The presence of antigenic cell components may interfere with or prevent agglutination of bacteria by homologous antiserum. The presence of Vi antigen in typhoid bacilli, for example, renders them inagglutinable with homologous O antiserum, but they become agglutinable as the interfering antigen disappears during successive subcultures. Similarly, colon bacilli contain a thermolabile component of the envelope antigen, the L antigen, which inhibits agglutination with O antiserum, but the bacterial suspension becomes agglutinable when this component is destroyed by boiling. This blocking effect of some antigens may be due to a spatial effect, such as steric hindrance, in preventing the primary union of antigen and antibody; or the inhibition may be one of the aggregation of sensitized cells to give agglutination in the secondary stage of the antigen-antibody reaction.

IMMUNOFLUORESCENT STAINING[6, 8, 15, 16, 17, 44]

The labeling of immune globulin by conjugation with fluorochromes was originated by Coons in 1942. It had been established that aromatic isocyanates may be coupled with protein by a carbamide linkage with free amino groups of the protein, commonly the ϵ-amino groups of lysine residues. This reaction is used to couple fluorochromes, most commonly fluorescein or rhodamine as their isocyanates, with immune globulin. The globulin is separted from the other serum proteins by the conventional salting out or methanol precipitation methods and reacted with the fluorochrome isocyanate, and the excess fluorochrome removed by dialysis. The antibody conjugate retains its antibody activity though some changes in other properties may occur.

Homologous antigen is specifically stained through the antibody activity of the conjugate, and may be in particulate form such as bacteria or vegetative virus, or as concentrations of soluble antigen. The application may be direct or indirect, *i.e.*, the antigen may be stained directly with homologous antibody, or indirectly by first treating the antigen with conventional homologous antibody and then staining by the application of fluorescent antibody to the antibody globulin as a variation on the antiglobulin technique described above. In any case, the specifically stained antigen fluoresces in ultraviolet light, requiring appropriate microscopic equipment for observation. Antigen stained with fluorescein conjugates fluoresces a yellowish green color, and that stained with rhodamine conjugates a reddish orange; both tend to fade on prolonged observation. There are a number of detailed descriptions of the foregoing techniques.[9, 18, 58]

While much less sensitive than the labeling of antigen or antibody with a radioactive isotope such as I^{131}, the fluorescent antibody method is much more flexible and has been invaluable in the localization of antigenic substances in the tissues, especially viruses which are otherwise not directly demonstrable.[51] It also has considerable value in the rapid identification of bacteria.[10, 58, 59] Nonspecific fluorescence often occurs but usually may be reduced to insignificance by preliminary absorption of the antibody, as for example with a brei of the tissue to be stained for contained homologous antigen.

ANTITOXINS

It was found by von Behring and Kitasato in 1890 that the immunity of rabbits and mice which had been immunized against tetanus was associated with the ability of the blood serum to neutralize the toxic substances produced by the tetanus bacillus. The substance in the serum which neutralized the tetanus toxin was designated by these workers as *antitoxin.* Subsequent investigation has shown that the animal body forms antitoxins in response to the injection of a variety of antigenic poisons, not only those of bacterial origin such as diphtheria toxin, botulinus toxin, and the like, but also against the phytotoxins and zootoxins.

The action of antitoxin may be directly demonstrated in the following way: if a fatal, or many times fatal, dose of toxin be mixed with an appropriate amount of antitoxic serum *in vitro,* the injection of the mixture into a susceptible animal is wholly without injurious effect; the poisonous qualities of the toxin are nullified by the immune serum. The reaction is, like all other immunological reactions, highly specific, and an antitoxin which neutralizes the homologous toxin is without effect on heterologous toxins.

The Toxin-Antitoxin Reaction. The combination of toxin and antitoxin does not necessitate the complete destruction of either component; neutral mixtures of toxin and antitoxin may be dissociated by treatment with hydrochloric acid, by freezing in the presence of phenol or tricresol, and, to some extent, by simple dilution. In certain cases, *e.g.,* pyocyaneus toxin and certain snake venoms, in which the toxin is more resistant to heat than the antitoxin, the latter may be selectively destroyed, and the neutral mixture becomes toxic upon judicious heating. It appears, therefore, that a more or less loose combination of toxin and antitoxin takes place, the poisonous properties of the toxin being neutralized as long as the union persists. The rate of reaction between toxin and antitoxin, like the chemical reactions, is dependent upon temperature, concentration, character of the medium in which the reaction occurs, and similar factors. The avidity of an antitoxin for its corresponding toxin differs in different cases; the union between tetanus toxin and antitoxin, for example, takes place less rapidly than that between diphtheria toxin and antitoxin.

An understanding of the precise character of the toxin-antitoxin reaction is dependent upon the interpretation of phenomena revealed by quantitative studies. It might be expected that a given quantity of antitoxin would always neutralize a constant amount of toxin, that the neutralization would follow the law of multiple proportions. This is, however, not the case, and it appears that the amount of antitoxin required to neutralize a given quantity of toxin is dependent upon (*a*) the manner in which the two are mixed with one another and (*b*) the relation between toxicity and combining power in the particular filtrate under consideration.

In the first instance it has been observed that when an excess of toxin is added to its specific antitoxin in several portions at proper intervals of time, much more unneutralized toxin remains in the mixture than if the same quantity of toxin had been added to the same quantity of antitoxin at one time. This is known as the *Danysz phenomenon.* If, on the other hand, antitoxin is added to toxin in successive equal portions, it may be shown that, in general, the first portion of antitoxin neutralizes a greater portion of the toxin than the second, the second a greater than the third, etc.

In the second case it has been found that there is no constant relationship between toxicity and combining power of a toxic filtrate; toxicity slowly diminishes upon storage but combining power remains unchanged. As described elsewhere (Chap. 9), the toxin is unstable and is slowly converted to toxoid. Both toxoid formed in this way, and that prepared by toxin inactivation with formaldehyde, retain their antigenicity and combine with antitoxin as well as stimulate the formation of antibody when

used as immunizing agents. Since the proportions of toxin and toxoid are not fixed, there is only an approximate correspondence between combining power and toxicity, and the toxicity neutralized by a unit quantity of antitoxin may vary from one filtrate to another or in the same filtrate at different times from 30 to 130.

The Standardization of Antitoxins. The quantitative evaluation of the toxin-neutralizing capacity of antitoxic serums is clearly a matter of considerable practical as well as theoretical importance. Ehrlich originally proposed as the standard unit of diphtheria antitoxin that amount which would just neutralize 100 guinea pig MLD's of toxin. As indicated above, however, variability in the relation of toxicity to combining power invalidates any standard based on toxicity; in other words, the combining power of a toxin is not a measure of its toxic qualities. On the other hand, since the combining power of a toxic filtrate remains constant within narrow limits, it is possible to establish an arbitrary standard unit upon which the relative strength of all antitoxic serums can be based. Such a standard diphtheria antitoxin was first prepared by Ehrlich and was preserved by him with all precautions against possible deterioration. A standard antitoxic serum based on Ehrlich's arbitrary standard unit is also prepared in this country by the National Institutes of Health of the United States Public Health Service, and is distributed every two months to the licensed producers of commercial serum. The unit is international, standard serums being tested from time to time under the auspices of the Expert Committee on Biological Standardization of the World Health Organization.[45]

Titration of neutralizing capacity. Three methods are used in the titration of diphtheria toxin and antitoxin. The first of these is the classic method of Ehrlich in which two limits (Lat., *limes*) are determined by guinea pig inoculation. These are the L_0 dose of toxin, which is defined as that amount exactly neutralized by the standard unit of antitoxin, and the L+ dose, that amount of toxin which, when mixed with 1 unit of standard antitoxin, is just sufficient to kill in 4 days a guinea pig approximately 250 gm. in weight. With these limits established, the serum to be standardized is mixed with the toxin just titrated, and the largest amount of serum which, when mixed with the L+ dose of toxin, will produce a 100 per cent mortality in the inoculated guinea pigs is considered to contain 1 unit of diphtheria antitoxin. This method is not mathematically sound as indicated elsewhere (Chap. 9), but the dose-response curve to the bacterial exotoxins is so steep that the procedure is practical and so persists.

A second method is based upon the observation that the intradermal injection of 1/250 to 1/500 MLD of diphtheria toxin into a guinea pig is followed by a local reaction, swelling and erythema and, with slightly larger amounts of toxin, necrosis, a phenomenon sometimes called the *Römer reaction.* By the use of such intradermal inoculations an Lr dose of toxin may be determined, *i.e.*, that amount of toxin which, when mixed with 1 standard unit of antitoxin, will produce the minimal skin reaction. The serum to be standardized is mixed in varying amounts with Lr doses of toxin, and that amount of serum which gives the minimal skin reaction is considered to contain 1 unit of antitoxin. This method has the advantage of allowing the testing of a number of toxin-antitoxin mixtures in one animal but has not displaced the classic method in common usage.

Flocculation. The third method is that of toxin-antitoxin flocculation, sometimes called the *Ramon flocculation,* and is a precipitin reaction in which the end point is the equivalence zone. Standard antitoxin is mixed with varying quantities of toxin and the tube first, *i.e.*, in time, showing precipitation contains one Lf dose of toxin. Varying amounts of the serum to be standardized are mixed with the Lf dose of toxin. The amount of serum in the tube first showing flocculation in this second series is considered to contain 1 unit of antitoxin.

The precipitation, or flocculation, curve is, as described elsewhere (Chap. 13), of the horse type in which the antigen-antibody complex is soluble in an excess of either reactant, and the Lf dose so determined is the optimal proportion zone of the reaction. This method differs from the other two in that it depends upon the combining power of a toxic filtrate rather than on toxicity. It is generally used, not as a final method of standardization, but as a preliminary to standardization by the Ehrlich method.

The inter-relationships of these limits are of some interest. The L+ dose is, of course, larger than the L_0 dose and, because of the peculiarities of the toxin-antitoxin reaction, by considerably more than one MLD. The Lr dose approximates the L_0 dose, as might be expected in view of the small amount of toxin required to elicit the skin reaction. The Lf dose is generally somewhat less than any of these, since it is a measure of combining power rather than toxicity and is unaffected by differences in the proportions of toxin and toxoid. It would appear that the Lf/Lr ratio should be the same for a given toxic filtrate immediately upon standardization. It has been found, however, that this ratio differs with different antitoxic serums because of variation in the avidity of antibody.

Antibody to Enzymes.[11, 40] The foregoing discussion of the toxin-antitoxin reaction applies only to the bacterial exotoxins, and diphtheria toxin in particular, *e.g.*, tetanus toxin does not flocculate as rapidly or sharply as does diphtheria or botulinum toxin. Other toxic activities of microbial origin, such as endotoxins, are only incompletely neutralized by antibody, and still others are enzymatic in nature or closely associated with enzymatic activity. The early hypothesis that bacterial toxins might be enzymes has not been borne out in the case of the exotoxins but, in a sense, seems to receive support from theories of the nature of the action of exotoxins such as diphtheria toxin (Chap. 9) and the enzymatic toxicities.

The way in which antitoxin serves to neutralize the activity of exotoxins in the toxin-antitoxin complex is not known. Some light may be shed on this point, and the partial neutralization of other toxicities, by indirection through the study of the inhibition of enzyme activity by antibody to the enzyme, for the enzymatic activity represents an activity of the molecule which may or may not be related to its antigenic character.

The degree of inhibition of enzymatic activity in the presence of specific antibody is a function of amount of antibody present. The rate of inactivation, usually titrated by adding increasing amounts of antibody to a constant amount of enzyme is at first linear, but the rate falls off, and in antibody excess a residual level of activity persists. The amount of residual activity is often related to the size of the substrate molecule, inhibition being substantially complete in the case of proteinases, collagenase, hyaluronidase, etc., and almost complete lack of inhibition is observed only rarely and when the enzyme has a low molecular weight substrate, *viz.*, β-galactosidase, tyrosinase, catalase, etc.

The inactivation may be only apparent in that aggregation of the enzyme in the antigen-antibody precipitate reduces its surface available to the substrate; such apparent inactivation is observed, for instance, with catalase. True inactivation appears to be a matter of steric hindrance, small substrate molecules penetrating between molecules of adsorbed antibody to the active sites on the surface of the enzyme, while large substrate molecules do not. This inference is not only supported by the general, though not invariable, relation of inhibition to size of the substrate molecule, but also by the varied consequences of the order and amount in which antibody and substrate are added to the enzyme, *i.e.*, antibody will not displace substrate, and by kinetic studies which indicate that antibody and substrate do not compete for the same reactive site.

It appears, then, that the site of enzymatic activity and that of antigenic specificity on the enzyme are not necessarily identical. In support of such an inference, cross-reactions between func-

tionally similar enzymes from widely different sources seldom occur, but they do occur between enzymes of closely related origin, and the latter is apparently attributable to antigenic relationships between the proteins rather than the prosthetic groups of the enzymes. While it seems clear that antigenicity and enzymatic activity need not be identical for inhibition of activity by antibody, it has not yet been possible to show unequivocally that the two are invariably not identical.

The analogy to the neutralization of toxin by antitoxin will be apparent at this point. The occurrence, or preparation, of toxoids from exotoxins, lacking toxicity but having unimpaired antigenicity, may be taken to suggest that the toxic portion of the molecule may be independent of its antigenic specificity. Similarly, the endotoxins are often said to be poor antigens since toxicity persists in the presence of excess antibody. They are, in fact, excellent antigens as assayed by the titer of, for example, precipitating antibodies they produce, but the antigen-antibody precipitate at the equivalence zone is toxic although no toxicity is detectable in the supernatant. In a sense, then, the neutralization of toxin by antibody may be only incidental to the antigen-antibody reaction.

LYSINS

It was observed by Nuttall in 1888 that freshly drawn and defibrinated blood, or serum, is markedly bactericidal for many kinds of bacteria and that this property is heat-labile and destroyed by holding at 55 to 56° C. for 30 minutes. Such heated blood or serum is said to be inactivated. This bactericidal effect is a consequence of the presence of natural antibody (Chap. 15) and possibly also properdin (Chap. 9) in the blood or serum, acting in conjunction with the thermolabile activity known as complement. The antibody concerned is cytotoxic or cytolytic and, because the lytic effect is the one most commonly made use of experimentally, such antibodies are lysins. In functioning as a lysin, the antibody mediates the effect of complement to give a secondary stage of the antigen-antibody reaction that is clearly set apart from the primary union or extensions of it.

Pfeiffer Phenomenon. One of the first demonstrations of the phenomenon of immune lysis was that by Pfeiffer in 1894 of the lysis of the cholera vibrio in the intraperitoneal cavity of the immune guinea pig. A heavy suspension of the vibrios is injected and samples of peritoneal fluid withdrawn from time to time and examined microscopically. The vibrios first lose their motility, swell up, and crumble into small fragments, and dissolution of even the fragments follows so no trace of the bacterial cell remains visible. This phenomenon may also be observed *in vitro* when the vibrios are mixed with antibody and complement.

Cholera vibrios and related microorganisms are relatively fragile, more so than most kinds of bacteria. Immune bacteriolysis may be observed with these microorganisms, and with certain others; for instance, some portion, but not all, typhoid bacilli so treated will lyse. The bactericidal effect, however, may occur without visible lysis, and it may be shown by appropriate serological methods that the cellular antigen has combined with both antibody and complement.[46]

Hemolysis. The lysis of bacteria by an immune serum is not a unique reaction in which only bacterial cells may play a part; bacteriolysis is, rather, a special case of a general phenomenon, for immunization with a variety of cells results in the production of cytolytic serums. Of these, erythrocytes have been by far the most widely studied, for the lysis of these cells, hemolysis, is readily apparent in the test tube; the red opacity of the cell suspension changes to the clear red solution of hemoglobin as lysis proceeds. The stroma is not dissolved but, upon examination, appears misshapen. First demonstrated by Bordet in 1898, hemolysis has been particularly useful not only as a type of lytic reaction

peculiarly suitable for laboratory manipulation, but also as an indicator of antigen-antibody combination when visible lysis does not or cannot take place. Such immune hemolysins, *i.e.*, those formed by the animal body in response to the injection of erythrocytes, are quite unrelated to the hemotoxins or hemolysins formed by microorganisms.

Complement.[25, 41, 49] The heat-labile activity participating in the lytic reaction is a constituent of normal serum, and its presence is unrelated to the immune response, *i.e.*, it does not increase in amount during immunization. This activity was called alexin by Bordet, in a restriction of the term as originally applied by Buchner to the undifferentiated heat-labile bactericidal action of blood, and complement by Ehrlich; the latter term is by far the more widely used.

Its status as a normal serum component is clearly indicated by the fact that lytic or bactericidal activity of freshly drawn serum, which has been destroyed by heat inactivation, is promptly restored by the addition of fresh normal unheated serum. There is little species specificity in complement; that from guinea pig serum, for example, may participate in the lysis of beef erythrocytes in the presence of antiserum prepared in the chicken. Some quantitative differences are apparent, for a serum which may be actively complementary in some reactions may be relatively inactive in other combinations, and the total activity is a function of the relative amounts of the various components of complement (see below) that may be present in a given serum.

Properties of complement. The property of inactivation by heat has been referred to above. Complement is also inactivated by shaking, but in neither case is the inactivation completely irreversible, for some activity may be regained on standing. In this respect complement behaves as a typical hydrophilic colloid which can be made to aggregate by physical forces but shows a tendency to spontaneous dispersion and restoration to the original state. The activity disappears upon standing—rapidly at room temperature (two or three hours) and more slowly in the icebox, where it may be preserved for three or four days. It is quite stable at very low temperatures in frozen form, and most of the activity persists after lyophilizing, and in lyophilized form it may be stored in a refrigerator for some months. Complement is irreversibly destroyed by strong acids or alkalis and reversibly inactivated by ions such as Mg, Ca, Ba, Sr, and SO_4 or by hypertonic salt solutions. In this connection it is of some interest that complement which is inactivated by raising the salt concentration to 5 to 10 per cent may be preserved at low temperatures in this form for several weeks, the activity being regained upon dilution with distilled water. These properties and some others, such as ready adsorption on surfaces, suggest a close relationship between complement and the enzymes; it has also been pointed out that there are remarkable resemblances to certain compounds of protein with soaps and lipids. An association of complement with the blood-clotting mechanism is suggested by the observation that many substances have both anticoagulant and anticomplementary activity, and that there is a close correlation between thromboplastic and complement-fixing power of tissue extracts and serums. It seems clear, however, that complement is not identical with prothrombin, and available evidence suggests that inactivation of complement of plasma blocks the conversion of prothrombin to thrombin. The nature of the lytic action of complement is as yet, however, purely speculative.

Complement is apparently rapidly formed in the intact animal; it has been found that, following apparently complete decomplementation of the guinea pig by the inoculation of egg albumin and homologous rabbit antiserum, appreciable complement titer is detectable at 8 hours and by 18 hours has returned to normal.

Nature of complement. Complement activity is intimately associated with the serum proteins and is inactivated by en-

zymatic digestion. The activity is not a property of a single substance, but of more than one differentiable substance. For example, when electrolyte is removed from serum by dialysis against distilled water, the euglobulin fraction of the serum proteins is precipitated, and a part of complement activity is present in this fraction and a part in the supernatant. Neither is active alone, but when they are recombined, activity is restored. The component present in the euglobulin fraction will unite with an antigen-antibody complex but does not produce lysis, while the soluble fraction will not combine with the antigen-antibody complex but with the antigen-antibody complex plus the euglobulin fraction. Because of this, the euglobulin fraction is known as the mid-piece of complement, and the other fraction as the end-piece. By a combination of various properties, complement, customarily designated C′, is separable into four fractions. These are:

C'_1: This fraction is the mid-piece described above which is precipitated by dialysis, or by bubbling CO_2 through serum diluted 1:10 with distilled water. It is heat-labile and is a euglobulin containing carbohydrate.

C'_2: This fraction is the end-piece and is also heat-labile. It is a mucoglobulin containing 10 per cent carbohydrate.

C'_3: This is a heat-stable fraction which is inactivated by treatment with cobra venom, yeast, or zymosan but is not inactivated by treatment with ammonia or primary amines.

C'_4: This is a heat-stable fraction which is inactivated by treatment with dilute ammonia or primary amines such as hydrazine, or by shaking with chloroform or ether, but is stable to treatment with cobra venom, yeast, and zymosan. It appears to be a carbohydrate portion of the mucoglobulin fraction containing C'_2, whose carbonyl groups are attacked by ammonia.

There is evidence that C'_3 may be resolved into two factors, C'_{3a} and C'_{3b},[54, 57] which may participate in successive steps in the hemolysis sequence (see below).[2, 53]

These components of complement are found in various animals but in differing proportions. For example, C'_2 and C'_4 are present in only small amount in the serum of the horse, mouse, cow, and sheep; sheep serum is also deficient in C'_3; and C'_2 and C'_3 are present in smaller amount than the other components in guinea pig and human serums. Since all four components are required for lysis, the complement titer of a serum is limited by the fraction occurring in smallest amount. Of readily available animal serums, that of the guinea pig usually shows the highest titer and is commonly used as a source of complement in serological reactions, either fresh or in lyophilized form.

The components of complement are titrated by using complement deficient in the fraction to be titrated. Such preparations are designated R′; and R'_1 is deficient in C'_1, R'_2 in C'_2, etc. The components tend to be functionally interchangeable to a considerable degree, as between human and guinea pig serum. On the basis of analyses of the complex, about 200 to 400 μg. of protein per ml. of serum combines with the antigen-antibody complex, representing the complement exclusive of C'_3 which does not combine, and the bulk of that which combines appears to be C'_1.

Mechanism of the Lytic Reaction. It is already apparent from the foregoing discussion that the lytic reaction involves three components, the antigen, the antibody, and complement. This is illustrated in the accompanying schematic

erythrocytes (antigen)	+ unheated immune serum (complement or alexin + amboceptor or sensitizer)	= hemolysis	
erythrocytes (antigen)	+ heated immune serum (amboceptor or sensitizer)	= no hemolysis	
erythrocytes (antigen)	+ heated immune serum (amboceptor or sensitizer)	+ unheated normal serum (complement or alexin)	= hemolysis

form for the so-called hemolytic system, *i.e.*, that in which the antigen is the erythrocyte. The three components of the system do not react with one another at random. The antigen-antibody reaction occurs first, and then complement, which does not react with either antigen or antibody alone, reacts with the complex. This may be shown in a number of ways. For example, if either antigen or antibody alone is mixed with complement, the complement activity remains free as demonstrable by appropriate titration methods (see below). Or, the antigen-antibody reaction occurs rapidly, even at 0° C., but complement unites with the complex only slowly at this temperature.[43]

It appears, therefore, that in immune lysis the antibody is not responsible for lysis, but rather mediates the action of complement as the lytic agent. Consequently, within this framework, antibody has been called the sensitizer, or substance sensibilisatrice, by Bordet and the French workers, and amboceptor by Ehrlich. Relatively small amounts of both antibody and complement suffice for lysis. Heidelberger has calculated that about 1500 molecules of rabbit antibody and 25,000 molecules of guinea pig complement are enough to lyse a single sheep erythrocyte. While figures such as these are no more than approximations, it is clear that only a very small part of the surface of the red cell need be affected to result in lysis.

The amount of antibody present is not a critical factor, but in regions of great excess of either antigen or antibody, lysis does not occur on the addition of complement. It is obvious that lysis will not occur if there is not enough antibody present. The failure of lysis in the presence of excess antibody is the phenomenon of complement deviation, or the Neisser-Wechsberg phenomenon, and is analogous to the prozone phenomenon in other serological reactions. The sensitizing action of antibody in the hemolytic system has a certain enzymatic character in that, in the presence of excess complement, antibody is transferred from one red cell to another, and lysis continues to occur slowly over a relatively long period.[42]

It is a matter of considerable interest that immune lysis may be passive. If, for example, a haptene is coupled with the erythrocyte by diazotization, or soluble antigen is adsorbed onto the surface of the red cell as in passive hemagglutination, such red cells are lysed by complement in the presence of antibody to the haptene or adsorbed antigen. Apparently, then, the combination of complement with the antigen-antibody complex in proximity to the surface of the red cell suffices to produce lysis even though the structure of the red cell is not directly involved in the initial antigen-antibody reaction. The way in which lysis occurs, in the case of the erythrocyte as leakage of the hemoglobin, is essentially unknown. On the basis of the dependence of inactivation of C'_2 and C'_4 by plasmin on the presence of C'_1 and calcium ions, it has been suggested[38] that C'_1 is a proenzyme which can be converted to an active enzyme by plasmin and the antigen-antibody complex, the latter serving as a catalyst in the activation. It is now definitely indicated that C'_1 is an esterase precursor which is activated during hemolysis, but its esterase activity appears to be independent of its activity as a component of complement.[4, 21] In this connection it is of interest that red cells may be sensitized to the lytic action of complement by treatment with substances such as colloidal silicic acid or tannic acid.

The reaction of complement with the sensitized cell is dependent upon the presence of both magnesium and calcium ions. In the case of lysis of sheep erythrocytes by Forssman antibody (it will be recalled that the sheep red cell contains Forssman antigen), the reaction with complement occurs in an orderly way.[5] The sequence of three reactions of the several components of complement with the antigen-antibody complex, the first of which requires calcium, and the second magnesium, ions. Using the symbols E = erythrocyte, and A = anti-

body, this sequence is represented by 1 below.

Complement Fixation.[48] Complement reacts with any antigen-antibody complex, including the precipitate from the precipitin reaction, toxin-antitoxin floccules, agglutinated bacteria, etc. When this reaction occurs, complement activity disappears in that it is no longer demonstrable in free solution, and the complement is said to be fixed. This is the complement fixation reaction or Bordet-Gengou phenomenon.

In systems in which the antigen is other than a lysable cell, there is no overt evidence that complement fixation has occurred, but the presence or absence of free, unfixed complement may be shown by the addition of an indicator system. This is a hemolytic system consisting of erythrocytes together with homologous antibody, and usually is sheep erythrocytes and homologous antiserum prepared in the rabbit. This provides the basis of the serological reaction known as the complement fixation test or reaction 2 below.

In this, as in other serological reactions, either the antigen or antibody may be unknown. If a known antiserum is used, an unknown microorganism or other antigen may be identified, and, conversely, serum may be titrated for antibody to a known antigen. This kind of serological reaction is especially useful when the antigen, known or unknown, particulate or soluble, may be difficult to prepare or obtain in purified form or in reasonably large amount, and is widely applied to viral and rickettsial antigens and antibodies.[23] It has been most familiar as the Wassermann test applied in the serodiagnosis of syphilis.

The question which is asked in the complement fixation reaction is the same as that asked in other serological reactions, *viz.*, do the antigen and antibody react? If union has occurred, complement is fixed, and its activity is no longer demonstrable in the reaction mixture by the hemolytic indicator system, and hemolysis does not occur. Conversely, if the antigen and antibody do not react, there is no antigen-antibody complex to fix complement, and hemolysis occurs in the indicator system. The specificity of this serological reaction lies, then, in the initial reaction between antigen and antibody, and the indicator system is nonspecific. For example, while the sheep erythrocyte and its homologous rabbit antibody are commonly used as the indicator system, the specificity of the complement fixation reaction would be unaffected if the rabbit erythrocyte and its homologous antibody prepared by immunization of the chicken were used.

The complement fixation reaction is technically more complex to carry out than many of the other serological reactions. It must be shown, for example, that neither the antigen nor the antibody is anticomplementary as used, *i.e.*, that neither alone reduces the activity of added complement. Similarly, the

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1.

$$E + A \longrightarrow EA \text{ (the initial antigen-antibody reaction)}$$

$$EA + C'_2 + C'_4 \xrightarrow{Ca^{++}} EAC'_{1\cdot4}$$

$$EAC'_{1\cdot4} + C'_2 \xrightarrow{Mg^{++}} EAC'_{1\cdot4\cdot2}$$

$$EAC'_{1\cdot4\cdot2} + C'_{3b} \longrightarrow EAC'_{1\cdot4\cdot2\cdot3b}$$

$$EAC'_{1\cdot4\cdot2\cdot3b} + C'_{3a} \longrightarrow E^* + \text{inactive product}$$

$$E^* \longrightarrow \text{stroma} + \text{hemoglobin}$$

2.

antigen + inactivated serum ← positive complement → negative sheep red cells + inactivated hemolytic serum (amboceptor)

amount of complement to be added must be determined by preliminary titration, and any residual complement activity in the serum to be tested must be removed by heat inactivation; too much or too little complement in the system will give false-negative or false-positive reactions respectively. In addition, the reagents must be added in the proper order. The antigen and antibody are allowed to react in the presence of complement for a sufficient time to allow the antigen-antibody reaction to occur and for the complex to react with, and fix, the complement. Then the indicator hemolytic system, red cells and homologous antibody, the latter also previously titrated, is added and allowed to react with any complement that may be present. Representative protocols for the complement fixation reaction and the required reagent titrations are given elsewhere (Chap. 2).

Quantitation. The amount of antibody present or, conversely, the ability of the antigen to react with the antibody, may be approximated by carrying out the complement fixation reaction with serial dilutions of the antiserum. It may be made more precisely quantitative by determining how much complement is fixed by the antigen-antibody complex. This is carried out by adding a precisely known excess of complement and back-titrating the unfixed complement with the hemolytic system.

The reaction is read as the amount of hemolysis and is inverse in that hemolysis is indicative of a negative antigen-antibody reaction. The end point may be taken as complete hemolysis, designated as ++++, and varying degrees of hemolysis as +, ++, or +++. The inverse relationship appears when no hemolysis is recorded as a ++++ antigen-antibody reaction, + hemolysis as +++ antigen-antibody reaction, etc. While this relatively crude criterion is sufficient for many purposes, the end point of complete hemolysis is subject to the same fallacy as a 100 per cent killing of microorganisms by a disinfectant (Chap. 6) or a 100 per cent mortality as an assay of virulence of toxicity (Chap. 9), *i.e.*, the sensitivity of erythrocytes to lysis is approximately normally distributed and 100 per cent lysis is a limit approached asymptotically. A more precise measure of hemolysis is, then, an interpolated 50 per cent end point from observed fractional hemolysis assayed photometrically.

OPSONINS

If a mixture of polymorphonuclear leucocytes and bacteria or other particulate matter is incubated for a time, it will be found on microscopic examination that a number of the leucocytes have ingested the foreign particles. Few if any particles will be ingested if the mixture is prepared in physiological salt solution, a considerable number if the fluid is normal serum. In the case of bacteria, great numbers of the microorganisms will be found packed into the leucocytes when the two are suspended in the specific immune serum. The antibodies present in the immune serum which so remarkably stimulate this engulfment by body cells are designated bacteriotropins, a term not in common use, or opsonins. The term opsonin was originally used to designate the activity of normal serum; hence the antibodies in the immune animal are sometimes called immune opsonins. The cells which ingest such particulate matter are termed phagocytes. This property is not confined to the polymorphonuclear leucocytes or heterophils, although these have been most widely used in *in vitro* experiments because of their availability, but is also present in various mononuclear phagocytes, both circulating and fixed tissue cells, or histiocytes, demonstrable in the latter by, for example, perfusion of isolated organs as well as *in vivo*.[32] These cells and their role in immunity are discussed in the following chapter.

The Opsonic Index. A quantitative estimate of opsonin present in a given immune serum may be made by comparing the number of bacteria ingested by

normal leucocytes in normal serum with the number ingested by normal leucocytes in the immune serum. The appropriate mixtures are prepared and incubated in capillary tubes, smears made and stained, and the bacteria engulfed by an arbitrary number (usually 50 or 100) of leucocytes counted. The average number of bacteria per leucocyte, or phagocytic index, is determined for the normal and immune serums, and the ratio of the phagocytic index of the immune serum to that of the normal serum is termed the opsonic index.

The opsonins may be estimated also by the dilution method; specimens are prepared in the usual way, except that the normal and patient's serums are diluted with saline or Ringer's solution. One mixture is made with salt solution or Ringer's solution to determine the degree of spontaneous phagocytosis. The dilution of serum which gives the same amount of phagocytosis as a mixture without serum is taken as the end point.

The Factors Influencing Phagocytosis. The process of phagocytosis is markedly influenced by environmental factors and the nature of the bacterium and leucocytes used, as well as by the amount of opsonin present. Departures from a neutral or very slightly acid reaction or from isotonicity, and the presence of certain ions, notably the citrate radical, depress the degree of phagocytosis. The last is of practical significance in that it contraindicates the use of citrated blood. The presence of calcium, on the other hand, may restore phagocytic power to leucocytes which have been allowed to stand in isotonic salt solution for a number of hours, a point of some interest in connection with the apparent intimate relation between this ion and cell division and the ability of ameboid cells to form new surfaces.

The nature of the bacterium to be ingested is of considerable importance; in general, virulent forms are relatively resistant to phagocytosis. It is not unlikely that this resistance is associated with the presence of a capsule; in the case of the pneumococcus, for example, not only are the smooth, encapsulated forms highly resistant to phagocytosis, but the presence of capsular polysaccharide markedly inhibits phagocytosis by an immune serum,[29, 56] presumably through combination with the antibody. Bacteriophage renders bacteria considerably more sensitive to phagocytosis.

The phagocytic power or activity of

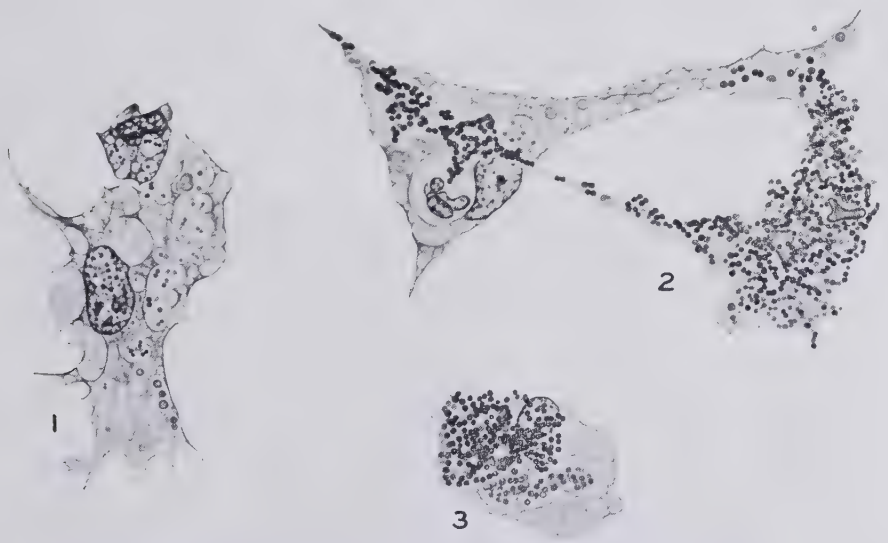


Figure 74. Phagocytosis of pneumococci by culture macrophages. *1*, Phagocytosis in presence of normal serum; relatively few bacteria have been ingested. *2* and *3*, Phagocytosis in the presence of anti-pneumococcus serum; enormous numbers of pneumococci have been ingested and appear as agglutinated masses. The lightly staining forms are degenerating. Hematoxylin and eosin-azure II. × 1200. (Zuckerman.)

the leucocytes is subject to considerable variation independent of variation in the opsonic content of the blood. This inherent phagocytic power of the leucocytes varies, with respect to certain bacteria at least, even in persons apparently in perfect health. In the child at birth the leucocytes are somewhat less active phagocytically than in the adult; they grow less active for a few months, and then more active, reaching the adult standard for streptococci, pneumococci, and staphylococci about the third year. In pneumonia, scarlet fever, and other conditions in which there is acute leucocytosis when the outlook is favorable, the phagocytic power of leucocytes has been found to be greater than normal for the specific bacteria. The increase in activity in such cases may be due to the predominance of young leucocytes.

The Process of Phagocytosis. The mechanism of ingestion is essentially one of the interplay of interfacial forces. Low interfacial tension of the bacteria against the leucocytes—and high interfacial tension against the medium—favors ingestion. The immune opsonins, and to some extent the "normal" opsonins, apparently form a surface deposit on the bacterial cells which promotes phagocytosis by altering the interfacial tension in this manner. In addition to surface tension, other factors such as the viscosity of the phagocytic cell substance enter into this phenomenon. Physical forces are not the sole controlling factors, however, for increased oxygen consumption accompanies the process of ingestion, the rise beginning at once, reaching a maximum value twice that at the start in about 15 minutes and persisting for 90 to 150 minutes.

It is of some interest that bacterial cells may be artificially "opsonized," *i.e.*, made more readily phagocytable, by treatment with iron ammonium alum, chrome alum, protamine sulfate, or gallotannic acid. The effect is reversed by treatment with oxalate, but reversion is very difficult when the cells are sensitized with immune opsonin. The significance of such observations in immune opsonization and phagocytosis is not clear.

Phagocytosis may occur quite as readily in the absence of antibody as in its presence, provided that it occurs on a suitable surface; most body tissues provide such surfaces, *viz.*, the phagocytosis of pneumococci on the alveolar surfaces of the lungs. This is the phenomenon of surface phagocytosis.[63, 64]

The Fate of Ingested Bacteria. Following phagocytosis many, but not all, species of bacteria may be observed to undergo a process of dissolution, with swelling, granulation, and fragmentation appearing as successive stages in their destruction. Although it was early supposed that intracellular digestion was no more than intracellular lysis through the agency of the immune lysin and complement which would have occurred

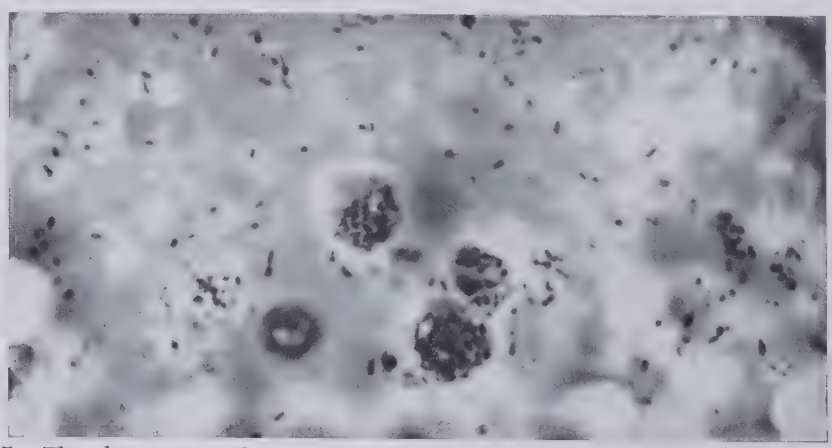


Figure 75. The phagocytosis of typhoid bacilli by leucocytes in whole blood. Note the enormous numbers ingested by the white cells and the bacilli lying free. Hastings' stain; × 1200.

whether or not phagocytosis took place, it now seems probable that the two processes are essentially different.

Bacteria are no exception to the rule that living organisms are not subject to attack by digestive enzymes, and the question arises as to whether death is a necessary preliminary to ingestion or whether it may occur within the phagocyte. It is probable that the viability of the microorganism is not an important factor in phagocytosis; in most cases the cell may be engulfed whether it is living or dead. In the case of those bacteria which are destroyed within the leucocyte, killing is necessary before digestion can take place.

Bactericidal agents are present within the phagocytic cells, but are not equally effective on all kinds of microorganisms; in some instances at least their activity appears to be potentiated by antibody.[24, 55] Such substances are designated leukins in the older literature; one, or a group, of these has been studied intensively and named phagocytin.[30] Others appear to be lysozyme-like in nature.[52] The efficacy of the killing process in the case of susceptible microorganisms is illustrated in observations[62] on avirulent streptococci, more than 90 per cent of which are destroyed in 15 minutes within the phagocytic cell. When these substances are not effective, the infected phagocytic cells serve to protect the microorganism from antibody[24] and may serve to disseminate the infection. Influenza virus, for example, is phagocytosed but remains infective for extended periods,[7, 28, 36] and in general virulent bacteria such as streptococci tend to persist and may destroy the phagocyte.

Leucocytes have been shown to contain a variety of digestive enzymes, including proteases, lipases, and various carbohydrate-splitting ferments which are presumably responsible for the actual dissolution of the engulfed particle.

The Nature of the Opsonic Activity. The opsonins are true antibodies in that they are increased by immunization and exhibit the specificity characteristic of the immune antibodies. The activity is, like that of the lytic serums, made up of two components, one thermostable and the other thermolabile. The thermolabile component is present in normal serum, and the reduced opsonic powers of an inactivated immune serum may be restored by the addition of a small amount of normal serum. In this and other respects the thermolabile component of opsonin strikingly resembles complement and in the past has been assumed by many to be identical with this component of the lytic system. In general, however, it has not been possible to show any constant relation between the components of complement and opsonization. For example, while the fourth component of complement has been reported to be necessary to the lytic reaction but not required for opsonization, it has also been observed that the thermolabile opsonin of the human serum is identical with C'_1, C'_2, and C'_4 in combination but not separately or in any combination of two. It appears, then, that complement and the thermolabile element of opsonin, while resembling each other closely in many respects, may not be regarded as identical.

PROTECTIVE AND NEUTRALIZING ANTIBODIES

When an antigen is a pathogenic microorganism, or a toxic product of a microorganism, antibody to it may protect a susceptible animal against infection or the effects of a toxic substance. Assay of this kind of antibody activity has been referred to earlier in connection with the standardization of antitoxins but is more widely applicable than indicated there.

The measurement of such antibody activity is dependent upon establishing a point of reference, such as the LD_{50} dose of microorganisms or toxic substance in the normal animal, and comparing with it a similar value obtained in the presence of antibody. In practice the challenge inoculum is ordinarily a multiple of the LD_{50} and, for sound mathematical

reasons, is held constant while the amount of antibody is varied.

The observed protective or neutralizing effect may be attributed to opsonic, lytic, antitoxic, and other antibody activities, and does not distinguish among them. At the same time, it measures the contribution of the antibody, and inferentially the antigen preparation, to effective immunity and thereby differs from the *in vitro* serological reactions. Such tests of antibody activity are of two general kinds, the protection test and the neutralization test, and the antibody activity so measured is said to be protective antibody or neutralizing antibody.

Protection Tests. The protection test is in turn subdivided into two kinds of tests, the active protection test and the passive protection test, depending upon whether the test animal forms its own antibody in response to the inoculation of an antigen or is the passive recipient of preformed antibody from another animal.

Active protection and immunogenic potency. The active protection test is a test of immunogenic potency of the antigen. It is carried out by inoculating groups of animals with serial dilutions of antigen, or a number, usually a fairly large number, of animals with a specified dose of antigen. The former is required for most kinds of experimental studies, and the latter is often used for the routine assay of the immunogenic potency of vaccines prepared and distributed commercially.

After a time, such as one to three weeks, during which the immune response develops, the animals are challenged with some standard dose, say 1000 LD_{50}, of the virulent microorganism or of the toxin. The protection conferred by immunization is measured as the amount of antigen required to protect 50 per cent, or some other specified portion, of the animals inoculated.

Since the details of this and other kinds of protection and neutralization tests are determined by the kind of microorganism or toxin used for immunization and subsequent challenge, such tests can be described in only general terms. At the same time, two points must be taken into consideration in tests of this kind. First, the susceptibility of the normal experimental animal, commonly the mouse, should be known to be reasonably constant, and animals of at least pen-bred homogeneity are usually required. Second, the microorganism must have relatively high virulence for the experimental animal for, when large doses must be used, those of living microorganisms required to kill may approach the toxicity level, *i.e.*, the lethal dose of killed microorganisms. This is generally true, for instance, in the case of the enteric bacilli, the gonococcus, the meningococcus, and certain other kinds of bacteria. The relative virulence of the microorganism thus affects the level of protection found; usually very high titer of protective antibody, of active or passive origin, is not observed when the microorganism is relatively avirulent.

Further, the relation of the experimental infection to the naturally occurring human disease needs to be taken into consideration when such experimentally determined immunogenic potency is used to characterize antigens intended for human use. For example, the virulence of the cholera vibrio for the mouse is very low, but may be greatly increased by suspending the bacteria in mucin to give a fatal fulminating bacteremic infection on intraperitoneal inoculation with perhaps 100 vibrios. The usual test for immunogenic potency of cholera vaccines is carried out in the mouse, but it is not clear what relation the challenge infection may have to the naturally occurring human infection in which the microorganisms are confined, for all practical purposes, to the lumen of the bowel and, therefore, to what extent immunogenic potency so measured is applicable to man. Similarly, the challenge inoculum of pertussis bacilli for testing active protection in mice is given intracerebrally. The infection would seem to be quite unrelated to whooping cough in man, but it is significant that protec-

tion of mice against infection by this route parallels that against infection by the intranasal route which produces an infection in mice similar to that observed in man.

Passive protection. In the passive protection test antiserum is tested for protective antibody by parenteral inoculation of groups of animals with graded doses in constant volume. Some time may be allowed to elapse between the administration of antiserum and challenge inoculation, 6 to 24 hours, so that the antibody is absorbed by the tissues of the animal. Or, if given nearly simultaneously, antiserum and challenge inoculation may be given by different routes, such as subcutaneously and intraperitoneally respectively. In any case, the passively immunized animals are challenged with some standard dose of the infectious agent or its products. The LD_{50} dose is interpolated in the usual way and, by comparison with the LD_{50} dose in the normal animal and the amount of antiserum required to protect 50 per cent of the immunized animals, the number of LD_{50} doses protected against by 1 ml. of antiserum, or its protective antibody titer, is readily calculated.

Neutralization Tests. The neutralization test is usually used in the neutralization of toxin by antitoxin and for the titration of antibody concerned in immunity to viral infections.[22, 39] It differs from the protection test in that varied amounts of antiserum are mixed with a standard dose of microorganisms or toxin, allowed to stand for a period, such as 30 minutes, to permit the antibody present to react with the antigen, and the mixture is then titrated for infectivity or toxicity. The assay may, of course, be carried out in the intact animal, but it is also applicable to tissue culture or egg culture while the protection type of test is not. The assay criterion is the death, or other effect, of 50 per cent of animals, tissue cultures, egg cultures, etc., and the titer of neutralizing antibody is calculated in the same way that protective antibody titer is calculated in the passive protection test.

REFERENCES

1. American Public Health Association: Diagnostic Procedures for Virus and Rickettsial Disease. American Public Health Association, New York. 1956.
2. Amiraian, K., Plescia, O. J., Cavallo, G., and Heidelberger, M.: Complex nature of the step in immune hemolysis involving third component of complement. Science, 1958, **127:** 239–240.
3. Becker, E. L.: Antigen-antibody reactions in gels. Fed. Proc., 1953, **12:**717–722.
4. Becker, E. L.: Concerning the mechanism of complement action. IV. The properties of activated first component of guinea pig complement. J. Immunol., 1959, **82:**43–51.
5. Becker, E. L.: Concerning the mechanism of complement action. V. The early steps in immune hemolysis. J. Immunol., 1960, **84:** 299–308.
6. Beutner, E. H.: Immunofluorescent staining: The fluorescent antibody method. Bact. Rev., 1961, **25:**49–76.
7. Boand, A. V., Jr., Kempf, J. E., and Hanson, R. J.: Phagocytosis of influenza virus. I. In vitro observations. J. Immunol., 1957, **79:** 416–421.
8. Borek, F.: The fluorescent antibody method in medical and biological research. Bull. W.H.O., 1961, **24:**249–256.
9. Cherry, W. B., Goldman, M., Carski, T. R., and Moody, M. D.: Fluorescent antibody techniques in the diagnosis of communicable diseases. United States Public Health Service, Publication No. 729, 1960.
10. Cherry, W. B., Thomason, B. M., Pomales-Lebron, A., and Ewing, W. H.: Rapid presumptive identification of enteropathogenic *Escherichia coli* in faecal smears by means of fluorescent antibody. 3. Field evaluation. Bull. W.H.O., 1961, **25:**159–171.
11. Cinader, B.: Antibodies against enzymes. Ann. Rev. Microbiol., 1957, **11:**371–390.
12. Cohn, M.: Techniques and analysis of the quantitative precipitin reaction. A. Reaction in liquid media. Methods Med. Res., 1952, **5:** 301–335.
13. Coombs, R. R. A., Coombs, A. M., and Ingram, D. G.: The serology of conglutination. Thomas, Springfield, Illinois. 1961.
14. Coombs, R. R. A., and Roberts, F.: The antiglobulin reaction. In Galton, D. A. G., and Goldsmith, K. L. A. (eds.): Haematology and Blood Groups, pp. 114–119. University of Chicago Press, 1961.
15. Coons, A. H.: Labeled antigens and antibodies. Ann. Rev. Microbiol., 1954, **8:**333–352.
16. Coons, A. H.: Histochemistry with labeled antibody. In International Review of Cytology, Bourne, G. E. (ed.): pp. 1–23. Academic Press, New York. 1956.
17. Coons, A. H.: The application of fluorescent antibodies to the study of naturally occurring antigens. Ann. N. Y. Acad. Sci., 1957, **69:**658–662.
18. Coons, A. H.: Fluorescent antibody methods. In Danielli, J. F. (ed.): General Cytological Methods, pp. 399–422. Academic Press, New York. 1958 .
19. Crowle, A. J.: Interpretation of immunodiffusion tests. Ann. Rev. Microbiol., 1960, **14:** 161–176.

20. Crowle, A. J.: Immunodiffusion. Academic Press, New York. 1961.
21. Cushman, W. F., Becker, E. L., and Wirtz, G.: Concerning the mechanism of complement action. III. Inhibitors of complement activity. J. Immunol., 1957, **79:**80–83.
22. Eckert, E. A.: Virus neutralization. Ann. N. Y. Acad. Sci., 1957, **69:**729–737.
23. Fulton, F.: The measurement of complement fixation by viruses. Adv. Virus Res., 1958, **5:**247–289.
24. Gelzer, J., and Suter, E.: The effect of antibody on intracellular parasitism of *Salmonella typhimurium* in mononuclear phagocytes in vitro. Prolonged survival of infected monocytes in presence of antibody. J. Exptl. Med., 1959, **110:**715–730.
25. Gorrill, R. H., and Hobson, D.: The properties of complement. Bull. Hyg., 1952, **27:**1–10.
26. Grabar, P.: Agar-gel diffusion and immunoelectrophoretic analysis. Ann. N. Y. Acad. Sci., 1957, **69:**591–607.
27. Grabar, P.: Immunoelectrophoretic analysis. Methods Biochem. Anal., 1959, **7:**1–38.
28. Hanson, R. J., Kempf, J. E., and Boand, A. V., Jr.: Phagocytosis of influenza virus. II. Its occurrence in normal and immune mice. J. Immunol., 1957, **79:**422–427.
29. Hirsch, J. G.: Immunity to infectious diseases: Review of some concepts of Metchnikoff. Bact. Rev., 1959, **23:**48–60.
30. Hirsch, J. G.: Further studies on preparation and properties of phagocytin. J. Exptl. Med., 1960, **111:**323–337.
31. Hole, N. H., and Coombs, R. R. A.: The conglutination phenomenon. I. An introduction to the conglutination phenomenon and an account of the observations and views of previous investigators. J. Hyg., 1947, **45:**480–489.
32. Howard, J. G., and Wardlaw, A. C.: The opsonic effect of normal serum on the uptake of bacteria by the reticulo-endothelial system. Perfusion studies with isolated rat liver. Immunol., 1958, **1:**338–352.
33. Ingram, D. G.: The conglutination phenomenon. XIII. In vivo interactions of conglutinin and experimental bacterial infections. Immunol., 1959, **2:**322–333.
34. Ingram, D. G.: The conglutination phenomenon. XIV. The resistance enhancing effect of conglutinin and immuno-conglutinin in experimental bacterial infections. Immunol., 1959, **2:**334–345.
35. Kabat, E. A.: Kabat and Mayer's Experimental Immunochemistry. Thomas, Springfield, Illinois. 2nd ed., 1961.
36. Köhler, H.: Die Rolle der Leukozyten als Virusträger. Zent. f. Bakt. I Abt., Orig., 1960, **180:**140–144.
37. Lamanna, C.: Adhesion of foreign particles to particulate antigens in the presence of antibody and complement (serological adhesion). Bact. Rev., 1957, **20:**30–45.
38. Lepow, I. H., Wurz, L., Ratnoff, O. D., and Pillemer, L.: Studies on the mechanism of inactivation of human complement by plasmin and by antigen-antibody aggregates. I. The requirement of a factor resembling C'_1 and the role of Ca^{++}. J. Immunol., 1954, **73:**146–158.
39. Mandel, B.: Neutralization of viral activity: Characterization of the virus-antibody complex, including association, dissociation, and host-cell interaction. Ann. N. Y. Acad. Sci., 1960, **83:**515–527.
40. Marrack, J. R.: Enzymes and immunology. In Sumner, J. B., and Myrbäck, K. (eds.): The Enzymes, vol. I, pt. I, pp. 343–389. Academic Press, New York. 1st ed., 1950.
41. Mayer, M. M.: Complement and complement fixation. In Kabat and Mayer's Experimental Immunochemistry, pp. 133–240. Thomas, Springfield, Illinois. 2nd ed., 1961.
42. Mayer, M. M., Levine, L., Rapp, H. J., and Marucci, A. A.: Kinetic studies on immune hemolysis. VII. Decay of $EAC'_{1,4,2}$, fixation of C'_3 and other factors influencing the hemolytic action of complement. J. Immunol., 1954, **73:**443–454.
43. McVickar, D. L., and Weinfeld, G. R.: Immune hemolysis at 0°. I. Introduction: The role of amboceptor and complement. J. Immunol., 1959, **82:**290–297.
44. Mellors, R. C.: Fluorescent-antibody method. In Mellors, R. C. (ed.): Analytical Cytology, pp. 1–67. McGraw-Hill, New York. 1959.
45. Miles, A. A.: The biological unit of activity. Its status and scope. Bull. W.H.O., 1949, **2:**205–213.
46. Muschel, L. H.: Serum bactericidal actions. Ann. N. Y. Acad. Sci., 1960, **88:**1265–1272.
47. Neter, E.: Bacterial hemagglutination and hemolysis. Bact. Rev., 1956, **20:**166–188.
48. Osler, A. G.: Quantitative studies of complement fixation. Bact. Rev., 1958, **22:**246–266.
49. Osler, A. G.: Functions of the complement system. Adv. Immunol., 1961, **1:**132–210.
50. Oudin, J.: Specific precipitation in gels and its application to immunochemical analysis. Methods Med. Res., 1952, **5:**335–378.
51. von Poetschke, G.: Der Nachweis von Viren und Virusantigenen mit Hilfe fluoreszenzmarkierter Antikörper. Progress Med. Virol., 1961, **3:**79–157.
52. Ralston, D. J., and Elberg, S. S.: Action of glycine and a lysozyme-like agent from rabbit monocytes in destruction of Brucella. Proc. Soc. Exptl. Biol. Med., 1960, **104:**464–467.
53. Rapp, H. J.: Mechanism of immune hemolysis: Recognition of two steps in the conversion of $EAC'_{1,2,4}$ to E*. Science, 1958, **127:**234–236.
54. Rapp, H. J., Sims, M. R., and Borsos, T.: Separation of components of guinea pig complement by chromatography. Proc. Soc. Exptl. Biol. Med., 1959, **100:**730–733.
55. Rogers, D. E., and Melly, M. A.: Further observations on the behavior of staphylococci within human leucocytes. J. Exptl. Med., 1960, **111:**533–558.
56. Suter, E.: Interaction between phagocytes and pathogenic microorganisms. Bact. Rev., 1956, **20:**94–132.
57. Taylor, A. B., and Leon, M. A.: Third component of human complement: Resolution into two factors and demonstration of a new reaction intermediate. Proc. Soc. Exptl. Biol. Med., 1959, **101:**587–588.
58. Thomason, B. M., Cherry, W. B., Davis, B. R., and Pomales-Lebron, A.: Rapid presumptive identification of enteropathogenic *Escherichia coli* in faecal smears by means of fluorescent antibody. 1. Preparation and testing of reagents. Bull. W.H.O., 1961, **25:**137–152.
59. Thomason, B. M., Cherry, W. B., and Pomales-Lebron, A.: Rapid presumptive identification

of enteropathogenic *Escherichia coli* in faecal smears by means of fluorescent antibody. 2. Use of various types of swabs for collection and preservation of faecal specimens. Bull. W.H.O., 1961, **25:**153–158.
60. Turk, J. L.: Immune-adherence with soluble antigens. Immunol., 1958, **1:**305–314.
61. Turk, J. L.: The immune-adherence activity of normal serum. Brit. J. Exptl. Path., 1959, **40:** 97–106.
62. Wilson, A. T., Wiley, G. G., and Bruno, P.: Fate of non-virulent group A streptococci phagocytized by human and mouse neutrophils. J. Exptl. Med., 1957, **106:**777–786.
63. Wood, W. B., Jr.: Studies on the cellular immunology of acute bacterial infections. Harvey Lectures, 1951/52, Ser. 47, pp. 72–98.
64. Wood, W. B., Jr.: Phagocytosis, with particular reference to encapsulated bacteria. Bact. Rev., 1960, **24:**41–49.

Chapter 15

THE IMMUNE STATE

Immunity to disease, or effective immunity operative in the specific immune state, is to be distinguished from the immune response in the technical sense on the one hand, and from resistance, sometimes called natural immunity or innate immunity, on the other. In the first instance, not all antigens provoke the formation of protective antibody. This is obvious in the case of antigens, such as ovalbumin, which are unrelated to pathogenic microorganisms or their products. Further, as implied elsewhere (Chap. 12), not all of the components of the complex of antigens making up the antigenic structure of a microorganism are concerned in effective immunity to the disease which it causes. Among the enteric bacilli, for example, antibody to the heat-labile antigens is apparently quite unrelated to effective immunity, and only antibody to the heat-stable antigens is protective.

Distinction between specific acquired immunity to disease and resistance or natural immunity is usually made on the basis of specificity, *i.e.*, acquired immunity is functional only against a specific microorganism, or sometimes a group of immunologically related microorganisms, while in natural immunity there is no distinction on the basis of antigenic specificity. The separation of acquired and natural immunity often cannot be made as sharply as implied. It is complicated by the common, practically universal, occurrence of so-called natural antibody, and the cellular response of acquired immunity differs quantitatively rather than qualitatively from that occurring in the normal animal.

The state of immunity to disease is separable into two parts: that which is primarily dependent upon the presence and activity of circulating antibody, humoral immunity; and cellular immunity, which is a function of the activity of phagocytic cells and the cellular response in the processes of inflammation. These parts also merge with one another, most obviously in the opsonic activity of antibody, in the function of cells in the synthesis of antibody in response to antigenic stimulus, and in the predominantly cell and tissue response of hypersensitivity mediated by antibody. Nevertheless, such a separation is useful for purposes of discussion.

HUMORAL IMMUNITY

The appearance of circulating antibody during recovery from infectious disease, and its association with the refractory state of specific acquired immunity, suggests that it may be casually related to effective immunity. The significance of antibody to effective immunity is firmly established by the kind of

experimental evidence which provides the basis of the passive protection and neutralization tests (Chap. 14). Less direct, but highly suggestive, evidence of the significance of circulating antibody in effective immunity is the high susceptibility to infection of individuals who fail to form γ-globulin. Congenital agammaglobulinemia, which may have a hereditary basis, has been observed in a number of instances[26] associated with susceptibility to generalized vaccinia and pyogenic infection. In general, it may be stated categorically that, so far as is known, acquired effective immunity rests upon an antigen-antibody basis.

Antitoxic Immunity. The function of various kinds of antibody activity in effective immunity is a matter that becomes partly speculative. As in the case of virulence of pathogenic microorganisms when virulence is attributable to essentially a single factor, such as toxigenicity and the pharmacological activity of the bacterial exotoxins, the nature of effective immunity to such a disease is equally clear. In tetanus and diphtheria, for example, the single, significant factor in acquired immunity is antitoxin, and antibody to the cell substance of the causative microorganism is, at the most, of only minor importance.

In the case of other soluble toxic products of pathogenic microorganisms, differentiation of the effective from the plausible function of antibody may be resolved to some extent by the protection test. The gaseous gangrene microorganism, *Clostridium welchii* type A, for example, produces three well-defined toxic products: the hemolytic α and θ toxins, and the κ toxin or collagenase. It might be supposed that collagenase activity would be a highly significant factor in a disease characterized by extensive tissue destruction and that antibody to it would be functional in effective immunity. This has not proved to be true, for antibody to the α toxin is protective while those to the other two toxins are not.

Or, antibody may affect the character of the disease but not otherwise materially modify its severity or prevent it. Scarlet fever is a clinical entity only because of the activity of the erythrogenic toxin, and antibody to this toxin prevents the appearance of the scarlatiniform rash, but contributes little or nothing to immunity to infection with the causative streptococci.

Antibacterial Activity. The function of antibody having other than antitoxic activity is less clear. The phagocytosis of microorganisms is a highly significant feature of immunity to infections with a variety of bacteria, such as the staphylococci, and it is highly probable that the opsonic activity of antibody plays an important part in effective immunity. In some instances, though, simple phagocytosis mediated, or accelerated, by circulating antibody may serve to protect the microorganism or even facilitate the spread of infection. Brucellosis, for example, persists in spite of high circulating antibody titer, and the inadequacy of the immune response, together with the limited effectiveness of chemotherapeutic drugs in this disease, is thought to be a result of persistence of the microorganism within host cells.

Agglutinins and precipitins would appear on the surface to serve no useful purpose in the defenses of the immune animal; for example, bacteria are not killed by agglutination. The clumping of bacteria and the precipitation of soluble foreign protein, however, considerably facilitate the process of phagocytosis, in that many bacteria may be engulfed at one time and insoluble masses of precipitated foreign protein may thus be removed.[11] In the case of pneumococcus antiserums, for example, protective antibody as measured by mouse assay appears to be identical with precipitin and agglutinin. The pneumococcus antibody has been found to enter pneumonic lesions in rats, agglutinating the free pneumococci in the alveoli and thus stopping the spread of the infection.[84]

In the absence of complement, microorganisms are not killed by antibody, and the not uncommon limited therapeutic effect of extremely high titer antibacterial serum has been regarded as an expression of the lack of sufficient

complement to take full advantage of the large amounts of antibody present. The bactericidal and lytic activity of complement mediated by antibody probably contributes significantly to effective immunity to at least some kinds of infections.

In some instances antibody may inhibit the reproduction of microorganisms. It was first suggested that antibody might function as an ablastin in pneumococcal infections, with suppression of proteolytic and glycolytic activity, but the evidence in this and other bacterial infections is inconclusive. In general, respiration is not affected by the presence of antibody, and an observed stationary microbial population may occur because the rate of reproduction is balanced by the death rate or because the microorganisms survive but do not multiply.

A reproduction-inhibiting effect of antibody has been demonstrated conclusively only in the case of the protozoan *Trypanosoma lewisi* where it is possible to differentiate between lytic activity and inhibition of cell division as attributable to different antibodies. A possibly similar mechanism is operative against the relapsing fever spirochetes (Chap. 33), but this is not conclusively established. Otherwise there is little evidence that inhibition of reproduction of the microorganism is a significant element in effective immunity.

Essential Immunizing Antigens. The relatively greater importance of antibody to some kinds of toxic substances to effective immunity may be expanded somewhat further. Antimicrobial antibody to various differentiable portions of the antigenic mosaic also vary widely in their contribution to effective immunity. In the case of pneumococcus, antibody to the capsular substance, *i.e.*, type-specific antibody, is primarily concerned in effective immunity to infection, while the contribution of antibody to the somatic antigen is relatively unimportant. In infection with this microorganism phagocytosis is a significant element of the defense mechanism, and the marked association between virulence of the pneumococcus and the presence of a capsule is a consequence of inhibition of phagocytosis by the capsule. Coating of the encapsulated bacteria with antibody facilitates phagocytosis so that antibody to the capsular substance is of primary importance to effective immunity. Or, in the case of streptococcal infection, antibody to the type-specific M antigen, which appears to be very superficially located on the cell, is of greatest importance to effective immunity, while that to the type-specific T antigen and to the group-specific C substance is of lesser importance. In the case of the enteric bacilli and the cholera vibrio, effective immunity is associated with antibody to the heat-stable somatic O antigen and that to the heat-labile H or flagellar antigen is not protective.[79]

In some instances the antigenic complex that stimulates the formation of protective antibody may be broken down further. As indicated elsewhere, the typhoid bacillus exists in two forms, the V form which contains Vi antigen, and the W form which does not. Vi-containing strains which lack typhoid bacillus O antigen can be obtained by dissociation, and certain other bacteria, notably a widely used strain of *Salmonella ballerup*, contain Vi antigen but are unrelated to the typhoid bacillus with regard to the specificity of the O antigen. Immunization with Vi-containing bacteria which lack the typhoid bacillus O antigen, whether typhoid bacilli or not, will protect against infection with V strains of the typhoid bacillus but not with W strains. Conversely, immunization with a W strain of the typhoid bacillus will protect against infection with either V or W forms of this organism, but not against infection with Vi-containing bacteria that lack the O antigen of the infecting strain. It is concluded, therefore, that antibody to the O antigen and that to the Vi antigen are concerned in protection. The components of the other O antigenic complexes occurring in the Salmonella group are not sharply differentiated in that all seem to contribute to an effective immunity.

The antigens so related to the effective immune response are sometimes spoken of as "essential immunizing antigens." It follows, of course, that not only should an effective immunizing preparation contain the significant antigens, but antigens extraneous to the development of an effective immunity may be omitted. The first consideration has indicated the use of typhoid vaccine prepared from Vi-containing strains, and the second is the basis of the endotoxoid vaccines such as those from the plague bacillus.

CELLULAR IMMUNITY

The cells of the body have two major roles in immunity to disease, the phagocytosis of microorganisms and other particulate material, and the formation of antibody. The former was established by Metchnikoff at the turn of the century, working first with the water flea, Daphnia, and then with mammalian cells. He differentiated two kinds of phagocytic cells, the microphages or polymorphonuclear leucocytes, and the macrophages or large mononuclear cells occurring both free and as fixed tissue cells. Although their immediate mobilization accentuates the limited significance of the polymorphonuclear leucocytes, the variety of fixed and free connective tissue cells of mesenchymal origin, grouped under the general head of macrophage, is of considerably greater importance in resistance to disease.

The distribution and inter-relationships of these phagocytic cells may be described briefly in outline form:[40, 75, 76]

(A) The predominantly fixed cells of the reticular and loose connective tissue which can be divided into two great groups:

(1) Fixed and free macrophages, including the reticular cells

(2) The fibroblasts of connective tissue and the endothelial cells lining the ordinary blood vessels.

(1) Macrophage is essentially a physiological designation for almost any large mononuclear connective tissue cell which is predominantly phagocytic and includes

(a) fixed cells such as

1. *pericytes* (Maximov), fixed, undifferentiated outstretched cells in the adventitia of all the small blood vessels of loose connective tissue throughout the body
2. *reticular cells* which, with fibers, form the stroma of all reticular (myeloid and lympathatic) tissues
3. *littoral cells* (Siegmund) which line the sinuses of the reticular tissues, liver, hypophysis, and adrenals. In the liver these are designated as *Kupffer cells*. Although frequently called endothelial cells or cells of the special endothelium, these are in fact either true reticular cells or have greater developmental potencies than ordinary endothelial cells.

These cells can divide by mitosis, become phagocytic, and develop into fibroblasts or practically any other blood or connective tissue cell. Here it is important that they can become phagocytic either in their fixed position (fixed macrophages) or after rounding up and becoming free (free macrophages).

(b) The free cells occurring in the loose connective tissue are variously known as *histiocytes, clasmatocytes, rhagiocrine cells,* or wandering resting cells, are either phagocytic or can become so without morphological change, can reproduce by mitosis, and transform into fibroblasts.

(2) The fibroblasts and endothelial cells are morphologically characterized by outstretched, ill-defined cytoplasm, a large oval vesicular nucleus containing dustlike chromatin granules, and small nucleoli.

(a) The fibroblasts divide by mitosis but do not develop into other cells (except in bone and cartilage) and are rarely phagocytic although instrumental in repair and walling off foreign material.

(b) The endothelial cells which line the larger blood vessels and capillaries (not including the littoral cells) likewise are rarely phagocytic but may be transformed into fibroblasts.

(B) The free connective tissue and blood cells.

(1) The cells of the blood and lymph are classified as to whether of myeloid or lymphoid origin:

(a) The *lymphoid cells* of the blood include the various sized lymphocytes which, together with monocytes, are termed *agranulocytes*. They divide mitotically and can develop into macrophages with all the latter's developmental potencies. As they become transformed into macrophages, they show increased amounts of cytoplasm, their nuclei take on macrophage characteristics, and they become phagocytic. These transitional forms are known as *polyblasts*.

(b) The *myeloid cells* are the various *granulocytes* (heterophils or polymorphonuclears, eosinophils and basophils), the erythrocytes, and platelets. Of these the heterophils are functional in immunity by virtue of their phagocytic activity but are "end" cells which do not reproduce or develop into other cells.

(2) The free mesenchymal cells are lymphoid cells indistinguishable from lymphocytes which occur in varying numbers in reticular and loose connective tissue and here act as precursors or "stem" cells of lymphoid and myeloid cells and hence may give rise to macrophages. They are variously termed *lymphocytes, hemocytoblasts, lymphoblasts, myeloblasts,* or *monoblasts* according to varying theories of blood formation. Under normal conditions lymphocytes in lymphatic tissue give rise only to lymphocytes, and hemocytoblasts in bone marrow only to myeloid cells, but under abnormal stimuli they may exhibit their full potencies for development. These stem cells are self-perpetuating, but as noted above, may arise from the fixed mesenchymal cells.

The Systems of Cells. It will be apparent that the cells primarily associated with defense against invading microorganisms are widely distributed through the body in the blood and lymph, cartilage, bone, reticular (blood-forming) tissue of the myeloid and lymphatic organs, and in the loose connective tissue associated with the skin, omentum, liver, lung, etc. It is customary to group the macrophages into so-called systems of cells. Metchnikoff's macrophage system includes the fixed tissue macrophages and the free macrophages and monocytes of the blood together with the various transitional forms, or polyblasts. Aschoff groups the cells concerned in defense as the reticuloendothelial system (RES) which, in the broad sense, includes the various fixed tissue macrophages and the free macrophages occurring in the inflammatory process. This term is unfortunate in that endothelial cells are not phagocytic, and the reference is to the so-called special endothelium such as littoral cells. The most all-inclusive term is the lymphoid-macrophage system which covers also the lymphoid cells which, as stem cells, may be transformed into macrophages.

Local Defense. The cellular response to an inflammatory stimulus is characterized by an initial migration of polymorphonuclear leucocytes to the point of injury. These cells are not numerous and soon disappear when the inflammatory material is sterile, but when bacteria are present they continue to migrate from the blood vessels and actively phagocytose the invading microorganisms. The functions of this first line of defense, although important, are strictly limited, since the cells are short-lived and do not multiply *in situ* but must be continuously recruited from the blood stream.

More important to local defense are the lymphoid cells of the blood and lymph, the lymphocytes and monocytes, which also migrate from the blood vessels but which, unlike the polymorphonuclear leucocytes, are long-lived and multiply in the tissues. These stem cells may be transformed to macrophages which, together with macrophages already present in the area, actively phagocytose and digest the invading microorganisms. When large bodies of foreign material are present, the macrophages may fuse to form foreign body giant cells; when microorganisms are indigestible, they may form giant cells around them such as the epithelioid cells of the tubercle. Their progressive development into fibroblasts supplies the active elements for regeneration and repair, the formation of scar tissue and the walling off of foreign bodies.

Certain other cells may take part in the late stages of the inflammatory process; the eosinophils, for example, may play a part in the detoxification of proteins and their disintegration products.

The inflammatory reaction[31, 42, 43, 69] plays an important part in eliciting this cellular response and contributes to the tissue injury produced. A polypeptide,

designated leucotaxine, which is found in exudate fluid, increases capillary permeability and provides a chemotactic stimulus for the early heterophil migration; its activity is antagonized to some extent by substances such as cortisone and hydrocortisone. A leucocytosis-promoting factor of pseudoglobulin nature, and found in the α-globulin fraction of exudate, is also produced in the inflammatory process that is associated with hyperplasia of granulocyte precursors in the bone marrow. A leucopenic factor, of polypeptide nature, is formed also, together with a toxic euglobulin called necrosin. The latter is absorbed from the inflammatory lesion, possibly producing injury elsewhere, especially in the liver and kidneys. This last is an example of a toxic substance of host rather than microbial origin as described elsewhere (Chap. 9).

General Defense. When a stimulant is distributed over a large part of the body the reaction is regarded as a general rather than local one; such distribution, however, usually means that the stimulant is in the blood stream and is combated by the cells of organs most closely associated with the blood, *i.e.*, the spleen, liver, and bone marrow, and in some cases general reactions may be regarded as local ones in strategically placed organs. The same types of cells are involved as in the local reaction, the polymorphonuclear leucocytes being mobilized first, with the macrophages playing the more important role. Endothelial cells, although in contact with blood-borne material, show very little phagocytic activity, and the fibroblasts are rarely active.

The Cellular Response in Immunity.[19] The above reactions, which are observed to occur in the nonimmune animal, are markedly accentuated in the immune animal, and there is an increase in the number of macrophages frequently designated as a hyperplasia of the reticuloendothelial system. The cooperative role of the humoral antibodies is of considerable significance; an antigen is localized by agglutination if cellular or by precipitation if in solution, and in either case the material is made more readily phagocytable by opsonization. Bacteria injected into the blood stream, for example, are rapidly removed in the immune animal by the fixed macrophages of the liver and spleen,[62] and staphylococci injected into the skin are localized and phagocytosed in great numbers. When antigen and antibody meet in the tissues of an immune animal, there are not only localization and opsonization of the antigen, but also a much heightened inflammatory reaction including a speeding up of the cellular response.

SITES OF ANTIBODY FORMATION[41, 53, 71]

Although antibody is ordinarily titrated and studied as it occurs in serum, serum antibody represents that synthesized by cells and spilled over into blood and body fluids. The function of cells in the synthesis of immune globulin is inferred from several lines of evidence, including interference with antibody formation by inhibition, removal or destruction of certain tissues, antibody production by transplanted cells, the distribution of antibody activity within the tissues, and the cytological events coinciding with antibody formation. While it is believed by some that both normal and immune globulin are synthesized in the same way, antibody resulting from a deviation in the normal synthetic processes, it does not necessarily follow that conclusions regarding the synthesis of immune globulin are completely applicable to the processes of synthesis of normal serum protein.

The relation of cells of the macrophage system to antibody formation is indicated by the results of interfering with their function. Blockade, by the inoculation of relatively large amounts of particulate material, such as carbon particles, so that all the phagocytic cells are packed with the substance and phagocytosis is sharply inhibited, markedly reduces the immune response. Similarly,

removal of tissue rich in the elements of the macrophage system, as by splenectomy, also markedly modifies the formation of antibody.

Gross injury to the cells of myeloid, as well as lymphoid, origin by ionizing radiation also affects antibody formation. When administered prior to, or within a short time after, the inoculation of antigen, irradiation sharply reduces or even eliminates the immune response, although a pre-existing immune response is not affected. The effect of lethal, or near-lethal, whole body x-irradiation is so marked that the homograft reaction disappears. Lethal doses of ionizing radiation destroy the bone marrow, and substantial protection is conferred by inoculation of the treated animal with bone marrow from normal animals. The irradiated animal does not give a homograft reaction to the transplanted cells, and, although they are eventually replaced when the animal survives, they persist and multiply with the appearance of blood cells of myeloid origin characteristic of the donor to give a radiation chimera. Irradiated animals similarly do not react to skin, tumor, and other tissue grafts.

If cells are primarily responsible for antibody formation, it would appear to follow that such cells would form antibody in tissue culture. It was reported in 1912 that cultures of guinea pig bone marrow and lymphoid tissue formed antibody to goat erythrocytes when the antigen was added to the tissue culture. In general, it has not been possible to repeat these observations; two reports[22, 67] of positive results have appeared, but confirmation is required in view of the many failures in the past.

It is well established, however, that antibody is formed in cultures of lymphoid tissue explants from previously immunized animals.[13, 66] It has been observed that the ability to continue to synthesize antibody is not necessarily dependent upon the integrity of the cells, and cell-free preparations of spleen taken from immunized animals have been found to form antibody.[68]

Such explants will also form antibody *in vivo, i.e.*, when inoculated into a normal animal.[51, 72] This occurs even when the recipient, such as the immature or irradiated animal, is unable to form antibody, or as in the case of the appearance of antibody to the typhoid bacillus in an individual having agammaglobulinemia but receiving lymphoid tissue from an individual immunized with typhoid vaccine.

Further, hypersensitivity of the delayed type (see below), which is not passively transferable by serum, may be transferred by the inoculation of a normal animal with lymphoid tissue from a sensitized animal. The occurrence of an immune response mediated by cells adds a third facet to acquired immunity (see below) which has been called "adoptive" immunity.[2, 14, 45]

The association of antibody formation with lymphoid tissue is of long standing, and there is an impressive amount of evidence in the immune response of the intact animal supporting it. For example, a number of workers, including more recently Erich and his co-workers and Harris and his co-workers, have carried out the kind of experiment in which minimal amounts of antigen are inoculated into the footpad of an experimental animal, usually the rabbit, and the afferent and efferent lymph from the regional lymph nodes examined for antibody activity. This kind of experiment is subject to control by giving different antigens on either side of the animal and titrating the lymph specimens for both kinds of antibody activity. In such experiments much more antibody to the locally inoculated antigen is found in the lymph draining from the node, the greater part of which is found contained in the lymphocytes. Such evidence is clearly indicative of the ability of lymphoid cells, but not necessarily mature lymphocytes, to form antibody.

In the same vein, the role of various organs in antibody formation may be examined. This is necessarily indirect because antibody-forming tissues apparently do not retain antibody. The importance of the spleen, for example, is indicated by the relatively large amounts

of antibody formed in tissue culture by explants from immunized animals, but the red pulp is more active than the follicular tissue.

The cytological evidence for the formation of antibody by cells is the localization of antigen and antibody within cells, and the cell types and changes associated with antibody formation.[12, 54] In the first instance, labeled antigens, *viz.*, coupled dye-proteins and antigens containing I^{127} or I^{131}, or S^{35}, may be used, or antigen may be located by treatment of the cells with fluorescent antibody. Similarly, antibody may be located within cells by treatment with fluorescent antigen. Studies of this kind have shown that antigen is found in phagocytic cells of the macrophage system, but that antibody is found, not in the same cells as antigen, but in plasma cells or immature plasma cells whose active nucleic acid synthesis is shown by their affinity for methyl green–pyronine.

In a general way, the immune response, especially the secondary or anamnestic response, is characterized by hyperplasia of the lymphoid-macrophage system. More precise studies have shown

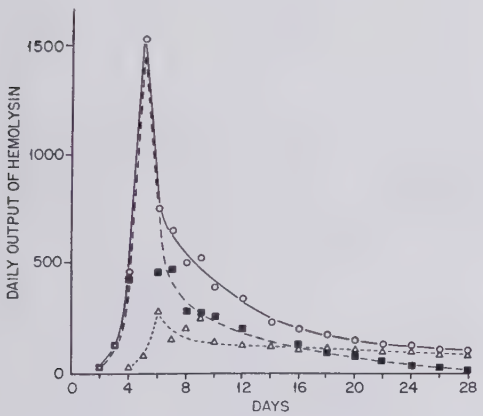


Figure 76. The mean daily output (not serum titer) of hemolytic antibody to the sheep erythrocyte by the rabbit following a single intravenous inoculation of antigen. The total output by the intact animal (circles), the nonsplenic output (triangles) by splenectomized animals, and the splenic output (squares) by difference are shown. Note the high peak and precipitous decline in splenic antibody output and the lower peak but continued production of nonsplenic antibody. (Taliaferro.)

that the antibody response is associated with the occurrence of the large pyroninophilic cells found to contain antibody. For example, in their studies on the rabbit lymph node, Leduc, Coons, and Connolly[38] found relatively few of these cells in the normal animal, but on antigenic stimulation larger numbers appeared in the medullary portion of the node, multiplied and differentiated, and accumulated increasing quantities of antibody in the cytoplasm until the development of the cells reached a point at which they appeared to be mature plasma cells. The primary and secondary immune responses were similar, but many more antibody-containing cells were found in the anamnestic reaction. A similar cellular response, but without identification of intracellular antibody, was observed in the spleen explants noted above, the red pulp explants containing large numbers of similar cells, described as "transitional," and the follicular tissue explants very few.[20, 32]

It has been found that single lymphoid cells, taken from an animal immunized with more than one antigen, form antibody to only one or another of the immunizing antigens, *i.e.*, individual cells form antibody of a single specificity and the observed multiple antibody response is apparently attributable to the pooling of the antibody output of many cells. This was observed as an agglutination of the bacterial antigen by antibody secreted by a single cell in an aqueous droplet in paraffin oil,[47, 48, 49, 50, 52] and has been demonstrated also in frozen sections of lymphoid tissue from immunized animals by the fluorescent antibody technique.[83] These observations have been taken to confirm the clonal selection theory of antibody formation (Chap. 13), but they are consistent with, rather than confirmatory of, this theory.

A general pattern in the relation of cells and tissues to the formation of antibody emerges from considerations such as the foregoing. The presence of antigen, or some fragment of antigen, within the antibody-forming cell is presumably required to provide both

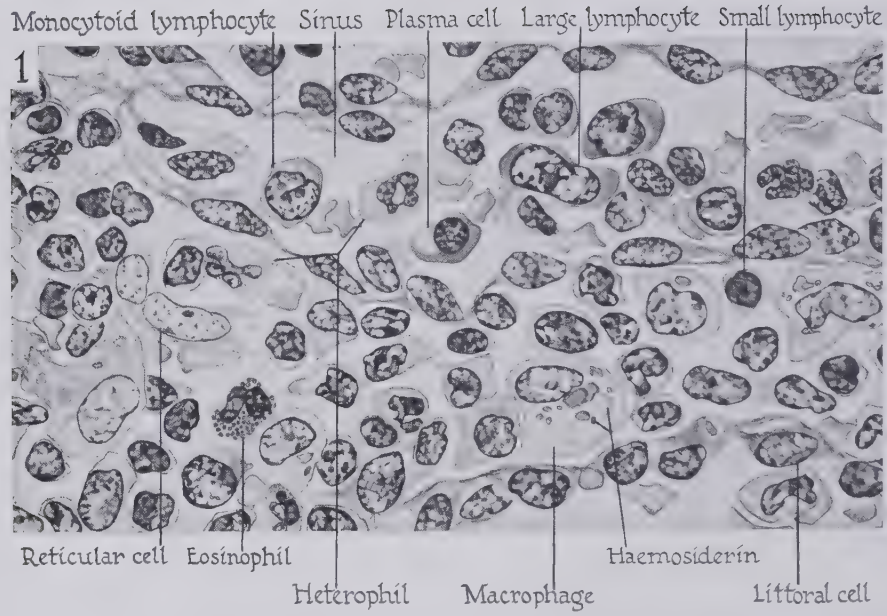


Figure 77. Cells of macrophage system. Sections of spleen of normal monkey showing a portion of a venous sinus and a Billroth cord in the red pulp; note the typical structure of the nongranular leucocytes, the reticular cells with indeterminate cytoplasm, and the rounded macrophages within the cord. × 1240. (Taliaferro and Mulligan.)

stimulus and template.[10] It seems not to be necessary, as once thought, that such cells be phagocytic. Apparently the first step in the sequence of events known collectively as the immune response is phagocytosis of the antigen which is then transferred, probably as a fragment and in some as yet not understood way, to the cells immediately concerned in the synthesis of immune globulin. This initial step is that which is interfered with in blockade and similar procedures, and the preparatory stage accounts, in part at least, for the induction period elapsing between inoculation with antigen and the first appearance of antibody, and is a radiosensitive stage in the immune response. Some breakdown of the antigen molecule is possibly the basis of the apparent requirement that the antigen be metabolized to some degree at least by the host tissues, and the rapid synthesis of antibody coincides with the disappearance of antigen in the blood.

The relative contribution of the antibody-forming mechanisms in various tissues to the total immune response is dependent upon the amount of antigen given and the route of its inoculation. When only small amounts of antigen are given other than intravenously, antibody tends to be formed locally, as in lymphoid tissue draining the area of inoculation, but large amounts of antigen given similarly spill out of the local area to stimulate antibody formation more generally. When the antigen is inoculated intravenously, the spleen is the site of early, and most intense, antibody formation, while antibody formation in other tissues tends to be somewhat less rapid but persists after the splenic response has declined.[16] While, then, the spleen is the most important source of antibody on appropriate initial antigenic stimulus, and as a consequence the antibody response is markedly affected by splenectomy, in the hyperimmunized animal the contribution of the spleen becomes less important, and antibody of nonsplenic origin becomes predominant.

Local Immunity. The defense mechanisms functioning in the immune state have been assumed to be general ones and, for all practical purposes, equally

effective throughout the body. It has been suggested, however, that these mechanisms are localized, or obviously accentuated, in certain tissues, not necessarily in tissues containing a large proportion of cells of the macrophage system, but tissues such as the skin, the intestinal mucosa, and the nasal mucosa.

Although attributable in some degree to the nature of the local defense factors, such as pH and the like, the predilection of an invading microorganism for some particular part of the body such as the central nervous system might be regarded as indicative of a relative susceptibility of certain tissues and, conversely, a relative resistance on the part of others—in Ehrlich's terminology, the presence or absence of cell receptors. In a disease such as typhoid fever or erysipelas, for example, it should be necessary then that only the intestinal mucosa or the skin be immune, the other tissues being already resistant. The evidence upon which it has been based is generally regarded as relatively weak, and local immunity in this sense probably does not exist.

Muco-antibody. There is, however, good reason to think that local immunity, not of a particular tissue such as the skin, no matter where it may be in the body, but of a local area, does exist, and is associated with local concentrations of antibody.[8, 57] Such antibody usually occurs admixed with mucus at mucous surfaces. That present in respiratory mucus reflects the rise and fall of serum antibody titer, and is considered to represent extravasated serum antibody. In other areas, notably the bowel and the vagina, such antibody titer does not parallel serum antibody titer, but reaches a peak earlier and subsides while serum antibody continues to rise. Local formation of antibody, indicated by a superior local response and markedly inferior serum response following local application of antigen, is consistent with this discrepancy, *i.e.*, the later peak in serum titer is a consequence of the cumulative nature of serum titer, while the local titer is a reflection of the rate of antibody formation.

Such antibody is called muco-antibody because of its occurrence at mucous surfaces, and in the case of that found in the bowel, coproantibody. A variety of antibodies have been described as occurring locally, including those to dysentery bacilli and cholera vibrios, to poliovirus, and to the α-toxin of *Cl. welchii* in the bowel, to Trichomonas in the vagina, and to leptospira in the urine.

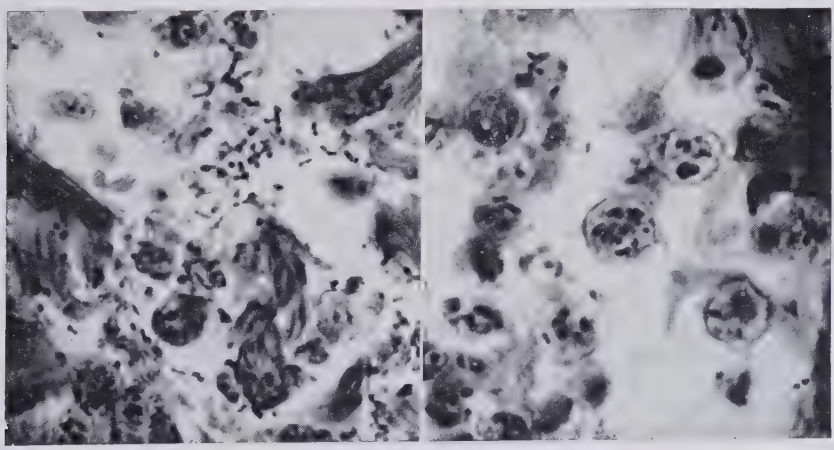


Figure 78. The cellular response in the immune animal. Rabbits injected subcutaneously with virulent pneumococci. *Left*, normal animal showing a typical spreading lesion with many extracellular pneumococci and minimal phagocytosis by polymorphonuclear leucocytes. *Right*, immune animal, showing marked phagocytosis with intracellular agglutination and few extracellular bacteria. Sections stained by Gram's method; × 1050. (Cannon.)

NATURAL IMMUNITY

As pointed out elsewhere, the defenses of the normal animal against infection are of two general kinds. The first, characterized by nonspecificity with respect to the infectious agent, has been called resistance and has been discussed earlier. The second kind showing a greater or lesser degree of specificity, is considered here as natural immunity.

It is the rule rather than the exception that pathogenic microorganisms are sufficiently closely adapted parasites that they are able to establish infection only in more or less closely related species of hosts. Thus it is perhaps not to be expected that the same parasite will be able to infect organisms as widely different as higher plants and mammals. It is of particular interest, therefore, that *Pseudomonas pyocyanea,* a not uncommon pathogen of man and higher animals, has been found able to infect tobacco plants and may be identical with the plant pathogen *Phytomonas polycolor.*

While in Ehrlich's terminology it might be said that the cellular organization of the plant lacks receptors for the human pathogen, there is evidence of a positive type of resistance to such infection. Antibiotic substances, similar to those produced by bacteria and fungi, have been found in a number of higher plants. Thus, extracts of cabbage, turnip, onion, and barberry plants inhibit the growth of coliform bacilli and *Bacillus subtilis*; chlorophyll and related compounds have been found to inhibit the growth of the tubercle bacillus; and substances which inhibit the growth of *Staphylococcus aureus* and coliform bacilli have been found in a large number of angiosperms. None of the plants, however, is known to produce anything resembling antibodies.

Specific Immunity. Natural immunity is often closely associated with the specific immune response, an immunity which is expressed in the form of the bactericidal and opsonic powers of normal blood, in the presence of the so-called normal agglutinins and the like. Such antibodies are generally present only in low titer, and the immunity associated with their presence in man is of a low grade but is possibly a significant part of that complex designated resistance. In many instances antibodies may be found under circumstances in which it is highly improbable that the animal has come in contact with the infectious agent; it has been noted, for example, that cattle serums will not infrequently neutralize the yellow fever virus and, more often, contain agglutinins for bacteria such as the typhoid and dysentery bacilli, the cholera vibrio, and certain of the rare varieties of Salmonella. Although present in low titer, such normal agglutinins appear to be no different from the immune agglutinins in adsorbability, heat resistance, and the like. The normal macrophage response does not involve this specific element, for all kinds of intact particles are phagocytosed. The question of the origin of these antibodies is, then, of some significance, and it may be asked whether or not specific antibodies may be formed in the absence of antigenic stimulation.

Natural antibodies. That the formation of some antibodies is genetically determined and takes place in the absence of antigenic stimulus is established in the case of the antibodies which determine the human blood groups. It is not unreasonable to suppose that in other instances the arrangement of polar forces on the surface of the normal globulin molecule might be such as to make possible to a greater or lesser extent the specific adsorption of antigenic substances. In fact, if the clonal selection theory of antibody formation (Chap. 13) is valid, serum globulin would contain antibody, at least in trace amounts, to all possible antigenic specificities. The probability that this has any such practical reflection, if indeed it does occur, is not great, and it is unlikely that such an explanation is generally valid.

Inapparent infection. A second pos-

sibility that accounts for the presence of normal antibodies is that of inapparent infection. There is no reason to doubt that the average individual is exposed more or less continuously to a wide variety of microbial antigens, beginning post partum and extending throughout life. The microorganisms constantly present in the form of normal flora, especially of the respiratory and gastrointestinal tracts, provide a source of antigens seeping into the tissues. The protective activity of pooled normal adult gamma globulin against a wide variety of infectious agents[44] is no doubt an expression of such antibody content of normal human serum.

A case in point is that of the appearance of diphtheria antitoxin in the blood in the absence of clinically recognizable infection; it is well known that the proportion of Schick-negative individuals increases in succeeding age groups. It is definitely established that the indicated formation of antitoxin is an immunity resulting from inapparent infection, *i.e.*, the carrying of virulent diphtheria bacilli in the throat, and the antigenic stimulus of the presence of small amounts of diphtheria toxin. The agglutinins for Flexner dysentery bacilli and some of the common species of Salmonella associated with food poisoning, not infrequently present in normal human serums to titers of 1:80 to 1:160, in all probability are a consequence of subclinical infections. It is probable that such a sequence of events occurs in a number of diseases and that so-called normal antibodies, such as the neutralizing power of normal adult serum for poliomyelitis virus, measles virus, and other infectious agents, are, in fact, immune antibodies. The manner in which a pseudoracial immunity may be produced through infection has been discussed elsewhere.

Common antigens. As indicated above, however, there are many instances in which it is extremely unlikely that the animal whose serum agglutinates a given bacterium came in contact with it either as a mild infection or otherwise. It is difficult to conceive, for example, of the ordinary cow in the United States coming in contact with the cholera vibrio. In such cases it is not necessary to assume either that the observed antibodies arose *de novo* through some genetic or maturation mechanism or that there was contact with the particular microorganism. It is only necessary to assume that the animal has come in contact with an immunologically similar antigen. In this case the cholera vibrio is immunologically related to Brucella, and brucellosis, in cattle or in man, results in the appearance of cholera agglutinins in the serum.[18] The likelihood that this will occur is clearly a function of the frequency with which immunological relationship or identity is shared by diverse organisms and the probability of contact with the unrelated form.

In addition to heterophile antigen, whose apparent random occurrence has been discussed elsewhere, immunologically similar antigens have been found to occur in a variety of seemingly unrelated organisms, and it is probable that many more have not as yet been discovered. For example, cross-reactions occur between type 2 pneumococcus polysaccharide and type B Friedländer's bacillus, certain species of yeasts, gum acacia and other vegetable gums, and some strains of coliform bacilli; the polysaccharide of type 14 pneumococcus is immunologically similar to the specific antigen of human blood group A; a constituent present in peptone is immunologically related to certain streptococcus antigens (group C); the capsule of the anthrax bacillus appears to be immunologically identical with that of *B. mesentericus*; the plague bacillus and paratyphoid B are immunologically related. From these examples it will be clear that immunologically similar antigens may be distributed in an apparently random fashion in unrelated organisms, and it is probable that in some, if not many, instances in which antibodies may be demonstrated for some microorganism with which infection or contact is unlikely, the antibodies are immune rather than "normal" and a consequence of exposure to a similar antigen.

In keeping with this discussion it may be pointed out that there is a strong possibility that there is no such thing as normal specific antibacterial immunity and that immunity in which a specific antibody may be demonstrated is one that arises as a consequence of exposure to the antigen. The incubation period following injection, for example, provides the opportunity for an immunological response that is difficult to rule out in many instances.

ACQUIRED IMMUNITY

Acquired immunity, as contrasted with natural immunity, is invariably an expression of a response to an antigenic substance. It is of two general kinds (in addition to the experimentally induced adoptive immunity described above), *viz.*, active immunity in which the immune individual forms his own antibody in response to an antigenic stimulus, and passive immunity in which the individual acquires preformed antibody produced by some other individual.

ACTIVE IMMUNITY

Active immunity is acquired as a consequence of exposure to the microbial antigen, either inadvertently as in infectious disease or inapparent infection as described above, or by deliberate inoculation with an appropriate antigenic preparation to give artificial immunization. The last is, as pointed out by Edsall,[17] an attempt to get something for nothing. The objective is an effective immunity to disease, not a simple immune response in the technical sense, and it is in this connection that the immunogenic potency of antigens is assayed by the active protection test (Chap. 14).

A number of immunizing preparations and methods of producing an active acquired immunity may be used. These are:

(1) the inoculation of living, fully virulent bacteria
  (a) rarely by a route favorable to infection
  (b) occasionally by a route unfavorable to infection
  (c) in conjunction with protective antiserum
  (d) which are drug-dependent
(2) the inoculation of attenuated bacteria of greatly reduced virulence for the host
(3) the inoculation of bacteria killed by
  (a) heat
  (b) antiseptics
(4) the inoculation of bacterial products
  (a) secreted during life
  (b) extracted from dead cells
(5) the inoculation of bacteria unrelated to the production of the specific infection

These may be briefly illustrated.

(1) Immunity produced by the introduction of living, virulent microorganisms practically identical with the immunity that results from an attack of disease after natural exposure. In experimental work the varying facility with which this mode of immunization can be effected is in part dependent upon the susceptibility of the organism to the particular parasite, and a highly susceptible animal can be immunized in this way only with great difficulty or not at all. The successful use of living cultures involves the administration of small nonfatal doses which are increased as immunity develops.

The relative insusceptibility to infection by some particular route may be taken advantage of, as in Ferran's method, now superseded, for protective vaccination against Asiatic cholera; natural infection occurs via the alimentary tract and subcutaneous injection of the virulent vibrios is followed by a local inflammation but not by a general infection with serious consequences.

The simultaneous administration of

virulent microorganisms and protective antiserum provides preformed antibody for combating the invader while allowing the immune reaction of the host to develop. This method is seldom used for the immunization of human beings but is common in some other instances; hogs may be immunized against hog cholera, for example, by simultaneous injection of virus-containing blood and antiserum. As has been described elsewhere (Chap. 7), strains of pathogenic bacteria may be made drug-dependent in that they cannot grow except in the presence of the antimicrobial agent, and are virulent only when the host is treated with the drug. When such strains are inoculated into susceptible animals, they are unable to produce infection and disease.

(2) As indicated elsewhere, the virulence of pathogenic microorganisms may be greatly diminished in a variety of ways, such as cultivation under unfavorable environmental conditions and by passage through animal species other than that of the host in question.[23] Perhaps the most familiar examples of the use of such attenuated material are those of smallpox vaccination and inoculation against rabies. In the first instance the virus of cowpox protects against smallpox infection, and in the second, rabies virus from dogs is "fixed," in Pasteur's terminology, or attenuated by serial passage in rabbits. This method is particularly useful in immunization against the virus diseases.

(3) Suspensions in physiological salt solution of bacteria killed by heating to 55° to 60° C. for 30 minutes or by treatment with formaldehyde or phenol are widely and successfully used in man against typhoid and paratyphoid fevers and against a variety of microorganisms in the laboratory. Such vaccines are, in general, most useful in infections in which the microorganism does not produce a soluble toxin but contains an endotoxin. This method has the obvious advantage of avoiding all danger of infection while at the same time introducing into the body the substances most intimately connected with the bacterial cell and its activities.

(4) The use of products of the bacterial cell for immunization purposes finds widest application in the case of those organisms which produce soluble toxins. As pointed out above, a solid immunity against diphtheria may be secured by stimulating the production of antitoxin. It is possible to build up such an immunity by using extremely small amounts of unmodified toxin in the early injections with gradual increases as immunity develops. In general, however, it is much more satisfactory to use neutralized or detoxified toxin. In the first instance a neutral mixture of diphtheria toxin and antitoxin (TAT) is frequently used with satisfactory results; the complex breaks down slowly in the body, liberating free toxin which acts as the antigen. Toxoid prepared by treatment with formalin which has lost its toxicity but retained its antigenicity is generally used at the present time in a partially purified form precipitated by alum. The alum-precipitated toxoid is of some advantage since the toxin-alum complex breaks down slowly in the tissues, liberating toxoid slowly to provide a prolonged antigenic stimulus.

As pointed out earlier, antibodies to certain portions of the antigenic mosaic of the bacterial cell contribute more to effective immunity than antibodies to other portions. Untoward reactions resulting from inoculation with the intact cell may be due in part to antigens unimportant to the development of effective immunity, and if these are eliminated from the immunizing preparation, the amounts of essential antigen may be increased. Considerations such as these have led to chemical and immunological fractionation of plague bacilli, for example, in efforts to prepare more effective immunizing antigens.

(5) Some degree of immunity toward specific infections may be developed by the use of certain kinds of bacteria or bacterial products entirely foreign to the infection in question. This protective effect seems to be of two general kinds. In the one, when the apparently unrelated microorganism contains antigens in common with the pathogenic form,

the protection conferred is that of the conventional antibody response. The other is transitory and a manifestation of the protective effect of endotoxins of the gram-negative bacilli against some kinds of pathogenic microorganisms. This phenomenon is well established and is, to a considerable degree, a consequence of a stimulation of the cellular defense mechanisms.[3, 34, 35, 63]

Irrespective of the antigen employed, the animal body requires time to respond with antibody production, and for a high degree of immunity successive antigenic stimuli are required. Early injections may consist of killed bacteria, followed by the inoculation of living, attenuated, or virulent cells. The process of forcing a very high degree of immunity upon an experimental animal is often termed hyperimmunization. The function of adjuvants in providing a persisting depot of antigen, and therefore a persisting antigenic stimulus to replace repeated inoculations, has been discussed earlier.

The immune response. The immunity produced is variable to some degree and dependent upon the efficacy of the antigen used. Some kinds of bacteria are good antigens, such as the typhoid bacillus, while others, such as the gonococcus, are poor antigens, and no effective immunity may be produced. The reason for this is not known. When an active immunity is produced, however, it is generally of long duration and effective over a period of years.

The immune response, as indicated by antibody titer, generally is apparent by the second of a series of injections, and the titer increases with succeeding injections until an upper limit is reached. With cessation of inoculations the antibody titer slowly declines and within a few weeks has reached a very low level. Subsequent injection brings about an immediate antibody response, much more rapid and pronounced than that of the initial immunization.

Polyvalent and multiple antigens. The high specificity of the immune response may require more than one strain or kind of microorganism to give effective immunity to a disease entity. Influenza, for example, is caused by a number of antigenically distinct strains of the virus with little or no cross-protection among them, and an immunizing preparation producing an immunity to the disease must include the relevant strains. Similarly, cholera vaccine often contains the two major serotypes of the vibrio, and typhoid vaccine may be combined with the paratyphoid A and paratyphoid B bacillus vaccines. Such immunizing preparations are known as polyvalent vaccines. The use of polyvalent vaccines is limited in practice by the number of components which are required. The total amount of antigenic material that may be administered without unduly severe reactions is limited. When, then, many components are required, the amount of each consistent with a tolerable reaction to the preparation may be so limited that no individual component is sufficient to provoke an effective immune response.

For the purely practical purpose of limiting the number of parenteral inoculations, it may be desirable to combine widely different immunizing agents as a multiple antigen. Commonly used combinations are, for example, pertussis vaccine and tetanus and diphtheria toxoids in some combination. The use of multiple antigens, and polyvalent vaccines, raises the question of whether or not the independent immune response to each of the antigenic components is adversely affected by the presence of the others, *i.e.*, whether the individual antibody responses are suppressed when they occur simultaneously. In some combinations there is some suppression of the immune response, but in others there may be an accentuation. The latter effect appears often to be due to an irritant effect of one antigenic preparation that accentuates the response to an accompanying relatively bland antigen. Various combinations of antigens behave differently, and the immunizing efficiency of multiple antigens cannot be generalized.[6, 27, 56]

Precocious protection. It has been observed from time to time that within a few hours after inoculation with an

immunizing antigen, the recipient develops an appreciable resistance to challenge inoculation which is usually, but not invariably, specific. For example, such a transient resistance develops rapidly after inoculation with typhoid or pertussis vaccine, in the latter instance reaching a peak in 8 to 10 days and prior to the appearance of demonstrable antibody. The phenomenon has also been observed with tetanus and diphtheria toxoids and with typhus fever rickettsiae.

This transitory protective effect is distinct from the active immune response and is usually regarded as an interference effect, based upon competition between the inactivated immunizing antigen and the challenge inoculum for tissue receptors. As such, it is closely similar to, if not identical with, the interference phenomenon among viruses (Chap. 4).

PASSIVE IMMUNITY

In contrast to active immunity, passive immunity involves no active generation of protective substances by the immunized animal. The latter is simply the recipient of antibodies formed in the body of another animal and transferred to the individual to be protected. Such passive transfer occurs in nature from mother to offspring, *in utero* via the placental circulation in man, apes, and rodents, in which there is only one layer of cells between the maternal and fetal circulatory systems, and after birth via the colostrum in ruminants, in which there are four layers of cells between the two circulatory systems.

The nature of the transfer of maternal antibody to the fetal circulation is not altogether clear. It is probable that the placenta does not play a significant part in antibody formation, and presumably it is a process of diffusion from the maternal circulation. Neonatal antibody may be considerably lower in titer than maternal antibody, and it has been observed that antibodies to different antigens are transferred to varying degrees, *viz.*, that to mumps and Japanese B encephalitis is relatively complete, while antibody to vaccina and poliovirus shows considerable disparity between maternal and fetal titers.[39] There is some reason to believe that incomplete antibody may pass from the maternal to the fetal circulation more readily than complete antibody.[46]

Absorption of antibody present in colostrum and milk occurs readily, at least in the immature animal,[4, 29, 30] and makes possible passive immunization of the newborn when transfer of antibody from the maternal circulation is not effective. Local formation of antibody occurs in the lactating mammary gland, and it appears to respond exceedingly rapidly to local antigenic stimulus. There is some evidence suggesting that local antigenic stimulus may be provided by microorganisms present in the suckling young, with consequent rapid return of antibody to the infant whose antibody-forming capacity is as yet poorly developed.[9]

Artificial passive immunization is brought about by the injection of immune serums such as the antitoxic serums of horses immunized against diphtheria toxin, tetanus toxin, and similar antigens, or of convalescent serums taken from humans who have recovered from the disease in question. As noted above, the γ-globulin separated from pooled adult plasma contains antibodies to the infectious agents encountered early in life, and is used for passive immunization of the young against infections such as measles. Passive immunization is most effective with antitoxic serums; with exceptions such as virus-neutralizing serums, antipneumococcus serums and the like, antibacterial serums are generally not highly effective either prophylactically or therapeutically.

Unlike active immunity, passive immunity is not of long duration, generally not more than two or three weeks. Repeated injections of tetanus antitoxin, for example, must be made at weekly intervals as long as the danger of infection remains. Horse serum globulin acts as a

foreign protein in the human body and tends to be eliminated as such. Passive immunization with homologous serum is possibly of somewhat longer duration; diphtheria antitoxin passively transferred *in utero* has been found to have a half-life of 4½ weeks.

HYPERSENSITIVITY

The response of the animal body to the presence of antigen in the tissues is clearly an advantage when the antigen is a toxin or the cell substance of a pathogenic microorganism. It was early apparent, however, that initial inoculation of an antigen may so sensitize the animal that subsequent inoculation results in the development of a typical symptom complex and perhaps death, and in such cases the animal is said to be hypersensitive. This effect is apart from such phenomena as the neutralization of toxin by antitoxin and is most striking when the antigen is initially bland. While it may play some part in effective immunity, there is also reason to believe that hypersensitivity to the cell substance of bacteria may, in some instances, contribute to the pathology of the disease resulting from infection as, for instance, in acute rheumatism and arthritis, and hypersensitivity to nonliving antigens may also result in disease of noninfectious nature. Hypersensitivity is quite general and may take a variety of forms in both experimental animals and man. It may be divided here into three general categories:

(1) anaphylaxis and related hypersensitivities
(2) allergy and/or atopy
(3) infection hypersensitivities

Of these, the first two are of the so-called immediate type in that reaction to the antigen occurs within a few minutes or hours, and the hypersensitivity is usually passively transferable by serum. They are distinguished from one another most obviously by the generally artificial nature of anaphylaxis, in a sense a laboratory phenomenon though it may occur under natural conditions, and by the spontaneous sensitization to environmental agents characterizing allergy and atopy. Infection hypersensitivities are somewhat set apart by their being delayed reactions, *i.e.*, reaction to the antigen is delayed one to four days, and by their failure to be passively transferred by serum.

ANAPHYLAXIS[15]

The sensitizing effect of an initial inoculation of antigen was observed by Richet in 1902 in a study of the immunization of dogs with toxic extracts of the sea anemone. About this same time Theobald Smith noted the lethal effects of second and third inoculations of diphtheria toxin-antitoxin in the guinea pig when the inoculations were widely spaced, and in 1905 Otto found that the active agent in the mixture was horse serum. This was subsequently studied in considerable detail by Rosenau and Anderson, and the general picture is now quite clear.

Anaphylactic Shock. If a guinea pig is inoculated with horse serum at intervals of perhaps four to eight days, it responds with the development of an immunity, and precipitating antibody appears in the serum. If, however, an interval of 10 to 12 days elapses between the first and second inoculations, the animal becomes hypersensitive as a consequence of the first inoculation, and the second is highly toxic. The state of hypersensitivity so induced by the *sensitizing dose* is designated *anaphylaxis*, and the reaction produced by the second, or *shocking dose*, is *anaphylactic shock*. This phenomenon of anaphylaxis is a laboratory artifact in that it results from

the purposeful inoculation of antigen into the tissues, and shock is produced by the rapid inoculation of relatively large amounts of antigen, usually directly into the blood stream. It is artificial in that it occurs only under conditions set up by man, in experimental inoculation of animals and in serum therapy in man.

The sensitizing dose may be exceedingly small, as little as 0.000001 ml. of horse serum in the guinea pig, and varies with different antigens and different experimental animals. The shocking dose is considerably larger, 0.1 to 0.01 ml. of horse serum for the guinea pig. Quantitative studies with a pure antigen, crystalline ovalbumin, have indicated that somewhat larger doses may be required. Guinea pigs sensitized with 10 μg. of ovalbumin could sometimes be fatally shocked with as little as 0.5 μg., and doses of 1 μg. gave fatal shock in about 40 per cent of the animals. Within a certain range the severity of the shock produced is directly related to the size of the shocking dose, *i.e.*, mild shock is produced by a small dose and lethal shock by a larger dose. The route of inoculation of the sensitizing dose is not important. For some animals and some antigens several sensitizing doses may be required to develop hypersensitivity, but in any case the doses must be small and widely spaced. A single dose suffices to shock, and the route of inoculation is important. Intravenous and intracardial inoculation is most effective, and, in the guinea pig, which is the most readily sensitized and shocked of the usual experimental animals, intraperitoneal inoculation of the shocking dose is effective, but the reaction is delayed and larger doses are required.

Once established, the anaphylactic state persists more or less indefinitely but diminishes appreciably in the guinea pig after a few weeks. If the shock produced is not lethal, the animal is temporarily refractory, that is to say, is *desensitized*, but hypersensitivity reappears, in the case of the guinea pig after perhaps two weeks or less. Guinea pigs, rabbits, and dogs are the common experimental animals, but birds may be sensitized, while mice, rats, and monkeys can be sensitized only with difficulty and then irregularly. The symptoms and postmortem pathology are essentially identical within a given animal species regardless of the antigen used, but differ from one animal species to another.

The guinea pig. Following inoculation of the shocking dose the animal remains quiet for a few moments but very soon becomes restless, the fur becomes ruffled, and the animal rubs its nose and sneezes. Clonic and tonic convulsions set in, the animal falls to its side, respiration becomes labored, and the animal dies gasping for breath while the heart continues to beat. This symptom complex is produced in varying degree in nonfatal shock. The outstanding postmortem findings are the marked distention of the lungs and hemorrhages on the underside of the diaphragm. The lungs remain distended after the thoracic cavity is opened and when removed from the body and cut. The bronchioles are sharply constricted with the retention of air in the alveoli, and the immediate cause of death is suffocation. The contraction of the bronchioles is not prevented by vagotomy or curare and is muscular or immediately neuromuscular in origin.

The rabbit. The rabbit is less susceptible to anaphylactic shock than the guinea pig, and, in contrast, develops shock only after a series of immunizing inoculations and after circulating antibody is demonstrable. Shock may be delayed and occur several hours after administration of the shocking dose. The symptoms and pathology differ sharply from those in the guinea pig with absence of difficulty in respiration. The animal falls on its side, shows convulsive movements, and passage of feces and urine. Respiratory movements may continue briefly after the heart has ceased to beat. On autopsy the pulmonary dilatation found in the guinea pig is absent, and the most conspicuous and characteristic feature is the extreme dilatation of the right side of the heart. The immediate cause of death is heart failure result-

ing from constriction of the branches of the pulmonary artery and consequent dilatation.

The dog. Anaphylactic shock in the dog occurs in two stages. The first is characterized by restlessness; the animal vomits and passes urine and feces and collapses with signs of extreme muscular weakness, and respiration is labored. The blood pressure falls rapidly as a consequence of capillary dilatation and stasis in the tissues. In nonfatal shock recovery from this stage is rapid, but in fatal shock the weakness is progressive, vomiting and diarrhea continue, convulsions may occur, and the animal becomes comatose and dies. On autopsy the outstanding feature is the congestion of the viscera and the pronounced distention and congestion of the liver, which is a consequence of constriction of the hepatic veins and injury to the liver cells resulting in edema from the transudation of fluid.

In addition to the characteristic phenomena noted above, anaphylactic shock also has common features which may be more or less pronounced in different animal species. Fall in body temperature is usual and leucopenia occurs which results from aggregation of leucocytes in the capillaries. Coagulation time of the blood is increased, usually most marked in the dog, as a result of the liberation of heparin rather than direct interference with the clotting mechanism, and serum complement is reduced. Congestion and hemorrhage of the gastrointestinal tract are common postmortem features.

Passive anaphylaxis. Anaphylactic hypersensitivity is passively transferable from sensitized female guinea pigs to offspring and by inoculation with serum from either sensitized or immunized animals. It can be produced with a high degree of consistency by inoculation of guinea pigs with immune rabbit serum and is a function of the amount of antibody, both precipitating and nonprecipitating, in the serum. As little as 0.03 mg. of rabbit or guinea pig anti-ovalbumin nitrogen suffices to passively sensitize guinea pigs, and univalent antibody is equally effective. The recipient is not immediately sensitized following the injection of antiserum and usually, though not invariably, a period of some hours elapses before sensitivity appears. If antigen is injected first, followed by the inoculation of antiserum, *reversed passive anaphylaxis* occurs, also usually some hours after the inoculation of serum.

Function of haptenes in anaphylaxis. The specificity of the anaphylactic reaction may be, and probably frequently is, determined by haptenes. In general, sensitization can be accomplished only by complete antigen, but in a few instances simple compounds, including arsphenamine, picryl chloride, and 2,4-dinitrochlorobenzene, have been shown to sensitize guinea pigs, and sensitization to arsphenamine has been observed in man. Haptenes will usually not elicit shock, though some of the larger molecules such as azo dyes will do so, but the inoculation of haptene specifically prevents shock by inoculation of the complete antigen. The role of haptenes in specificity is further shown by the use of conjugated antigens containing the same haptene but different proteins; thus, guinea pigs sensitized with a conjugated globulin can be shocked with the corresponding conjugated albumin.

Anaphylactic shock in isolated tissue. It will be clear from the foregoing discussion that the occurrence common to anaphylactic shock in different animals is a contraction of the smooth muscle, predominantly in the bronchioles in the guinea pig, in the pulmonary artery in the rabbit, and in the hepatic veins in the dog, and in all three the gastrointestinal tract is affected. It is, then, of considerable interest that anaphylactic shock, manifested as a sharp and abrupt contraction, can occur in isolated smooth muscle. This was observed by Schultz in 1910, using portions of intestine, and studied in detail by Dale with uterine horn, and is now generally known as the *Schultz-Dale technique.* The uterine strip is suspended in a bath of Ringer's solution with one end tied fast and the other attached to a kymograph, and the

contractions are recorded when antigen is added to the bath. The reaction is exquisitely sensitive, a sensitized strip giving typical contraction in final dilutions of horse serum antigen as high as 1:1000 million, and sharply specific. All of the essential features of anaphylaxis may be reproduced *in vitro* by this method, including active or passive sensitization of the uterine strip *in vivo*, passive sensitization *in vitro*, specific desensitization, etc. It is significant too that uterine horn from immunized as well as sensitized guinea pigs will respond to antigen with shock.

Mechanism of anaphylactic shock. It will be clear that anaphylaxis rests on an immunological basis and that shock is the result of an antigen-antibody reaction. There is good reason to believe that the union is one of antigen with intracellular or sessile antibody. For instance, as just indicated, sensitization and shock may be produced in isolated tissue, and the incubation period in passive sensitization is regarded as necessary for the taking up of inoculated antibody by the cells. Desensitization consists of saturating, or nearly saturating, sessile antibody, and the protective effect of immunity is a prevention of antigen reaching intracellular antibody by union with serum antibody. Antigen-antibody complexes have pharmacological activity, and soluble complexes prepared with excess antigen will produce various manifestations of hypersensitivity, including anaphylactic reactions, skin reactions (see below), etc., in the normal intact animal or isolated tissue.[60, 78, 80, 81]

The manifestations of anaphylactic shock are closely similar to those produced by histamine and substances having histamine-like activity. In the guinea pig, for example, constriction of the bronchioles is produced which is indistinguishable from that of acute anaphylactic shock, and histamine and similar substances produce contraction of isolated smooth muscle in the Schultz-Dale reaction. It is generally believed that the antigen-antibody complex formed affects the cells and tissues in a way, as yet unknown, that results in the release of histamine-like substances. There is no reason to believe that antibody is fixed primarily or even predominantly in smooth muscle cells, but there is a high correlation between histamine sensitivity and the cells concerned in anaphylactic shock. Furthermore, the tissues affected in anaphylactic shock are those that show the highest content of histamine; there is some evidence which suggests that leucocytes are a source of histamine and, as indicated above, they aggregate in affected tissues in shock. In addition many, though not all, substances showing antihistamine activity appreciably or markedly modify anaphylactic shock; of these epinephrine and ephedrine are among the most effective.

While the activity of histamine and its resemblance to anaphylactic shock, and other manifestations of hypersensitivity, have been known for a long time, it is probable that other substances are concerned also. For example, antihistamines are only partially effective in inhibiting anaphylactic shock; the increased clotting time characteristic of anaphylaxis is not produced by histamine; refractoriness to histamine is not necessarily associated with refractoriness to anaphylactic shock, etc. Of other histamine-like substances, serotonin (5-hydroxytryptamine) has also been associated with anaphylactic reactions. For example, both histamine and serotonin are released when antigen and antibody are added to a plasma suspension of normal rabbit platelets[36] and occur in rabbit blood during anaphylactic shock. The possibility that the predominant substance may differ from one animal species to another is suggested by the high content of histamine in the guinea pig lung which contains little serotonin, and the reverse relationship in the mouse lung,[82] and the partial protection of guinea pigs but not mice by some antihistamines, while lysergic acid diethylamide, a serotonin inhibitor, protects mice.[21] That more than one histamine-like substance may be effective in a single animal species is suggested not only by the incomplete protection given

by antihistamines, but also by the observation that smooth muscle from an animal sensitized with multiple antigens reacts to each antigen successively, *i.e.*, the contractile stimulant is not exhausted in one reaction.

A possible neurogenic mechanism in hypersensitivity reactions has been of interest in connection with the role of nerve conduction in the Schultz-Dale reaction. For example, stimulation by antigen or serotonin, but not by acetylcholine or histamine, is inhibited by structural analogues of serotonin such as gramine, yohimbine, and bufotenine, suggesting that the antigen-antibody reaction may liberate serotonin. Treatment of the muscle preparation with botulinum toxin (Chap. 9), however, blocks stimulation by antigen but not by serotonin, which has been taken to indicate that the toxin blocks the release of serotonin.[24]

The nature of the pharmacological reactions mediated by the antigen-antibody union is clearly a highly complex one.

The Arthus Phenomenon. The Arthus phenomenon is a local hypersensitivity reaction produced in actively and passively sensitized animals, which is closely related to, but not identical with, anaphylaxis, and which has been called a form of local anaphylaxis. If rabbits, for example, are injected repeatedly with antigen such as horse serum, a local reaction appears which becomes more and more intense as the inoculations are repeated. The site of inoculation first shows a transient swelling, but later the swelling and edema persist and progress to induration and local necrosis. This local phenomenon is a result of a general sensitization since later inoculations need not be in the same site as earlier ones. The Arthus phenomenon appears to be confined to the rabbit among experimental animals, or at least is very difficult to produce in other animals, but occurs with some frequency in man, as during protracted series of inoculations with antiserum or an antigen such as rabies vaccine.

The rapidity of the reaction and its intensity are correlated with the amount of precipitable circulating antibody as well as with the amount of antigen inoculated. The reaction is an immediate one in that a cellular response is demonstrable histologically within an hour but it does not become grossly apparent for several hours. The local antigen-antibody reaction results in the deposition of the complex in the tissue adjacent to the site of inoculation, with preferential location in and on either side of the basement membranes of the small blood vessels.[64] Such localization undoubtedly contributes to the vascular necrosis which is a prominent feature of the histopathology of the lesion. When a dye is inoculated with the antigen, it diffuses rapidly in the skin of a normal animal but is localized when the animal is sensitized; this reaction is refered to as passive cutaneous anaphylaxis (PCA) when used for the detection of skin-sensitizing antibody.[55] The Arthus reaction may also be reversed by the administration of antigen, followed several hours later by the local inoculation of antibody.

Serum Sickness. The inoculation of man with antiserum, usually from the horse, produces in some persons a characteristic syndrome termed serum sickness. The symptoms include rash, often urticarial in nature, fever, joint pains, some edema, and swelling of the lymph glands regional to the site of inoculation, in combinations and emphasis that are variable from one individual to another. This reaction is to be distinguished from the febrile and local reactions commonly following the inoculation of foreign protein and is most often attributable to a hypersensitivity to horse serum irrespective of its antibody content, though essentially similar reactions may be produced following the inoculation of toxoids, vaccines, etc., as a consequence of hypersensitivity to constituent antigen. The incubation period may be as short as two hours or as long as 24 days, and most often is 8 to 12 days. The reaction may follow initial inoculation

of horse serum, but more frequently there is a history of prior inoculation. It is specifically antagonized by epinephrine, ephedrine, etc., and, like anaphylactic shock, is presumably a consequence of the sudden liberation of histamine in toxic amounts.

The hypersensitive individual gives an immediate reaction, *i.e.*, appearing within 10 to 20 minutes, to the intradermal inoculation of 0.1 ml. of 1:10 dilution of horse serum or the instillation of a drop of horse serum in the conjunctival sac. In the first instance an irregular wheal surrounded by an erythematous zone constitutes a positive reaction, and in the second diffuse conjunctivitis appears in the hypersensitive individual.

Under certain circumstances, such as the prophylaxis of tetanus with antitoxin, desensitization may be carried out relatively rapidly. The usual procedures, after a preliminary test for sensitivity by intradermal inoculation of 0.02 ml. of a 1:10 or 1:100 dilution of the serum, include the administration of drugs such as diphenylhydramine hydrochloride, epinephrine, and corticotropin by intravenous drip, and, after 30 minutes or so, small amounts, 0.1 ml. of a 1:100 dilution, of antiserum are given subcutaneously. Successive inoculations can be given at relatively short intervals, 10 to 15 minutes, and doubled with each inoculation. Eventually 1 ml. doses can be given every 15 minutes until the total dose of antitoxin has been administered.[37, 73]

The question may be raised as to why serum sickness rather than anaphylactic shock occurs in the hypersensitive individual. In general there seems to be little tendency in man to generalized shock in this and other manifestations of hypersensitivity, and, as in dogs and monkeys, there is a tendency to localization of the manifestations of the reaction to the respiratory tract, the gastrointestinal tract, and the skin. A number of instances of typical anaphylactic shock have been reported in man though it is rare, and when generalized shock occurs it is often fatal.

ALLERGY AND ATOPY

Serum sickness is perhaps best regarded as a form of anaphylaxis in man, but only a small part of the manifestations of hypersensitivity are those of serum sickness; the remainder may be grouped under the general head of allergy. Like anaphylactic shock, an allergic reaction is a consequence of the union of antigen with sessile antibody. The antigen is often designated *allergen* and the corresponding antibody *reagin* because it was thought earlier that they differed from antigen and antibody. It is now generally recognized that there are no essential differences, but the terms persist even though a number of workers have urged that they be dropped.

There are a number of differences between anaphylaxis and allergy which are rather of degree than of kind, but which in the aggregate tend to distinguish them. Thus, allergy is naturally acquired while anaphylaxis is artificially produced; allergy is often a hypersensitivity to nonprotein antigens while anaphylaxis is only rarely so; allergic hypersensitivity is of long duration while that of anaphylaxis is limited; desensitization is usually difficult and incomplete in allergy and effective in anaphylaxis; and edema is a prominent feature of the allergic reaction and smooth muscle contraction a minor factor while the reverse is true in anaphylaxis.

Some workers divide the clinical allergies into two general groups: *atopy* (strange disease) or *atopic allergy*, and *nonatopic allergy*. The atopic allergies include the pollen and dander sensitivities resulting in hay fever and asthma and some of the food and drug allergies. Contact dermatitis and the remainder of the food and drug allergies make up the nonatopic group. The distinction between the two is quantitative rather than qualitative, and it is doubtful whether it is of any real validity, but it does have a certain clinical utility. It was first made on the basis of heredity, hereditary predisposition being an important factor in atopy, and it has even

been postulated that in atopy initial contact with the antigen is not necessary for the development of the allergic state. On the other hand, in the nonatopic allergies there is almost always a history of contact with the antigen, such as continued exposure to nitrocellulose products in industry, and the hereditary predisposition seems to be of minor importance. There are other correlated characteristics. In atopic allergy the sensitivity is very high, desensitization is difficult and usually only partial at best, skin reactions are marked and specific, and considerable amounts of antibody are demonstrable in the serum. In nonatopic allergy the converse generally holds, *i.e.*, sensitivity is low, desensitization is usually successful, skin reactions are weak and nonspecific, and little antibody is demonstrable in the serum. These distinctions are, however, purely relative.

Heredity. It has been a matter of some interest that inheritance of a predisposition to at least some forms of allergy is an important, perhaps often a determining, factor. The constitutional factor is a predisposition only, and contact with the antigen is essential to the development of the allergic state. Inheritance in man is frequently difficult to demonstrate, but detailed studies on familial association have made it highly probable that predisposition to allergic hypersensitivity is genetically determined. It has been suggested that the predisposition is determined by a pair of allelomorphic genes, H determining nonallergy and h allergy. The possible genotypes are HH or pure normal, hh determining allergy which develops before puberty, and Hh the normal transmitter in which allergy may develop after puberty. In confirmation of observations on man, the inheritance of predisposition to sensitization has also been shown in experimental animals. The significance of heredity in allergy has been said to distinguish this form of hypersensitivity from anaphylaxis, but it is not clear that this is a basis of distinction.

Forms of Allergy. The allergic state is manifested in a variety of forms which are determined by two inter-related factors, the portal of entry of the antigen and the tissue predominantly affected, usually referred to as the *shock organ.* Thus the antigen may be inhaled, ingested, injected, or may simply make contact with the skin. The tissues affected are those of the upper respiratory tract, the gastrointestinal tract and the skin. These combinations give rise to a number of commonly occurring, well-defined clinical types, *viz.*:

Hay fever. This is a seasonal allergy produced by the inhalation of pollens from trees, grasses, and weeds, and time of occurrence is determined by time of pollination, *i.e.*, trees and grasses from late spring to midsummer and weeds in late summer and early fall. In the United States ragweed pollen is one of the most common offenders. Nonseasonal hay fever is a result of hypersensitivity to animal danders, orris root (a constituent of many cosmetics), and house dust. The mucous membranes of the upper respiratory tract are affected primarily.

Asthma. Essentially the same inhaled antigens are responsible for asthma as for hay fever, and in addition book bindings, straw, and similar materials are sometimes involved. The individual may become sensitive to bacteria of the normal flora of the upper respiratory tract, giving rise to so-called endogenous asthma. The shock organ is the lining and musculature of the bronchi in bronchial asthma, by far the most common type, and swelling of the mucosal lining and spasm of the muscles results in obstruction of the smaller bronchioles and consequent difficulty in breathing. Allergic asthma may also result from the ingestion of foods, such as eggs, milk, wheat, or various drugs. It may be noted that asthma is a symptom complex and not always allergic in etiology.

Dermatitis. Dermatitis of allergic etiology may result from contact with the antigenic substance or from ingestion or inhalation of antigen. The former is frequently referred to as *contact dermatitis* and is often an occupational disease resulting from repeated contact with substances such as lacquers, nitrocelluloses, glue, and the like. Ingested antigen not infrequently affects the skin as the shock organ, giving rise to a *noninfectious eczema* in infants and what is often called *neurodermatitis* in the adult.

Urticaria and angioneurotic edema. When the skin is the primary shock organ an inflammatory reaction accompanied by some degree of edema results. Urticaria, or hives, is the occurrence of whitish or pink elevations which come and go repeatedly within short periods, and is the most common lesion. In angioneurotic edema, or giant urticaria, edema is much more pronounced, and the lesions are large pale swell-

ings which cover areas such as the eyelids, lips, and genitals. Hives and this edematous kind of lesion are frequently found together and most often result from hypersensitivity to ingested or injected antigen, *i.e.*, foods and drugs.

The foregoing clinical types are characterized by the symptom complex produced, but types of allergy may also be differentiated on the basis of kind and portal of entry of antigen, *viz.*:

Drug idiosyncrasy. As indicated above, an allergic antigen may be a simple chemical compound, and when this is a drug the condition is a drug allergy or drug idiosyncrasy. The allergic sensitivity is to be differentiated from the sensitivity of a low tolerance for the drug; in the first instance the symptoms are those of the allergic reaction, while in the second they are produced by the pharmacological action of the drug. The drugs commonly involved are the barbiturate derivatives, salicylic acid compounds, phenolphthalein, opiates, sulfonamides, occasionally the antibiotics, the arsenicals, and others. These are either ingested or injected. The most common reactions are urticaria and dermatitis, and bronchial asthma is less frequent.

Food allergy. The ingested antigens include food as well as drugs, and strawberries, milk, and eggs are the most common offenders. The shock organ in food allergy is usually the skin, and the most frequent lesion dermatitis or urticaria, and, less often, bronchial asthma. In addition, the gastrointestinal tract is often affected directly with resulting disturbance.

Pollen and dander allergies. These antigens are inhaled, and the symptoms are almost always those of involvement of the upper respiratory tract, most commonly hay fever, and bronchial asthma somewhat less so.

Contact allergies. This is almost entirely the contact dermatitis noted above and is not only an occupational disease, but not infrequently results from cosmetics, the lacquers such as nail and hair lacquers, and powders containing orris root.

It will be clear from the foregoing that it is difficult if not impossible to generalize to a satisfactory degree the various inter-related forms of allergy, and the source of the difficulty is that they are not fundamentally different.

Allergic Antigen and Antibody. As indicated above, the allergic antigens or allergens (sometimes called atopens in the atopic allergies) are frequently nonprotein in nature. This is most obvious in the case of the drug idiosyncrasies in which synthetic substances, such as barbiturates, antipyrine, and the like, are clearly not contaminated with protein. Similarly, the substances responsible for contact dermatitis, such as lacquers, are protein-free. The more complex naturally occurring allergens such as pollens, danders, and foods are, of course, not protein-free, but the active agent in some pollens is of lower molecular weight, perhaps 5000, than the usual antigenic proteins. There is no doubt that haptenic substances can produce the allergic reaction in a sensitized individual, at least in experimental anaphylactic shock, but the question of sensitization is more difficult. High molecular weight nonproteins can act as complete antigens; for example, sensitization may be induced by pneumococcal polysaccharide. It is probable, however, that the relatively simple substances, such as the arsenicals, do not function as sensitizing antigens in themselves, and it is generally believed that they combine with body protein to give a conjugated complete antigen whose specificity is determined by the haptene, which functions as the sensitizing antigen. It is not clear, however, whether the shocking antigen is such a conjugated one or whether the haptene alone usually produces shock.

It may be noted here that allergic hypersensitivity to physical stimuli, heat, cold, and light, may occur. It is unlikely that such stimuli alter body proteins so that they become iso-antigens, and such allergies probably do not have an immunological basis.

On intradermal inoculation of soluble antigen, or its application as a patch on the intact skin, the sensitized individual gives a skin reaction characterized by local erythema and the appearance of a wheal. The skin reaction is more readily elicited and more specific in some allergies than in others. In general it is not satisfactory in contact dermatitis and some of the food and drug allergies. This kind of skin test is to be distinguished from tests like the Schick and Dick tests, in which the reaction is produced by diphtheria or scarlatinal toxin and neutralized by circulating antitoxin, which have no relation to hypersensitivity.

Antibody is demonstrable in many of

the allergies, particularly those grouped under the head of atopy. Its presence may be shown directly in some instances by complement fixation, but the usual measure is passive transfer of the sensitivity. If a small amount, 0.1 ml., of serum from a sensitized person is inoculated intradermally into a nonsensitive person and the antigen inoculated intradermally in the same area 24 hours later, a positive reaction occurs. The local sensitization may persist for four weeks or more. Such passive transfer cannot be made to guinea pigs but has been made to rhesus monkeys, and it is probable that a close phylogenetic relationship is essential. This passive transfer was described by Prausnitz and Küstner in 1921 and is known as the *Prausnitz-Küstner reaction.* A reversed passive sensitization, analogous to reversed passive anaphylaxis, may be produced by inoculation of antigen first, and serum 24 hours later. In the usual terminology, the antibody which produces passive sensitization is the allergic reagin, but other antibodies which do not sensitize have also been described. The development of more than one antibody is, of course, to be expected when the antigen contains a mosaic of specificities.

As in the case of anaphylactic shock, the allergic reaction is a consequence of the union of antigen with sessile antibody which results in the liberation of histamine or histamine-like substances. Allergic shock is antagonized by histamine antagonists, such as epinephrine and ephedrine, and a number of synthetic histamine antagonists, derivatives of dialkylaminoethoxy compounds (Benadryl and Decapryn), dialkylaminoethylamines (Antergan, Antistine, Pyribenzamine, Neohetramine, Phenergan), and dialkylaminoalkyl compounds (Trimeton, Thephorin).

HYPERSENSITIVITY IN INFECTION

Bacterial cell substance may act as a sensitizing as well as an immunizing agent, and anaphylaxis may be induced with bacterial protein, though usually with much greater difficulty than with highly antigenic proteins such as egg albumin and serum proteins. Sensitization may also occur in experimental and naturally acquired infection but is highly variable, being especially prone to occur with some bacteria and not with others.

Hypersensitivity to the tubercle bacillus is the best known of the infection hypersensitivities and is typical of them. It is demonstrable as a delayed (one to four days) local inflammatory skin reaction, the tuberculin reaction, to preparations of soluble antigen of the tubercle bacillus known as tuberculin. The tuberculin reaction is considered in detail elsewhere. This kind of reaction is somewhat different from the immediate wheal type of reaction, with respect to time of development, the nature of the dermal reaction, and in that it is not passively transferable. The generalized response also differs from the immediate shock of anaphylaxis; the inoculation of a tuberculous guinea pig with tuberculin in a dose sufficient to kill does not result in death until after some hours, and on autopsy the site of inoculation is congested, focal glands are swollen and congested, and focal reactions occur about tuberculous lesions which consist of areas of enormous dilatation of the capillaries. This is obviously different from fatal anaphylactic shock in the same animal. The nature of the antigenic stimulus inducing tuberculin-like sensitivity has been shown by Raffel[25, 58, 59] to be a protein combined with a wax fraction of the bacilli, the protein alone giving the usual immune response with the formation of precipitins.

The infection hypersensitivities differ from anaphylaxis and allergy in that they are not passively transferable by serum. This kind of hypersensitivity is, however, passively transferable as shown by Chase; the inoculation of a normal animal with cells from an induced sterile peritoneal exudate in a sensitized animal confers the hypersensitivity on the recipient. This transfer is analogous to the adoptive immunity produced by cell transfer described above.

Hypersensitivity is the outstanding im-

munological response to infection with a number of other microorganisms, and the skin reactions have been of some interest from a diagnostic point of view. In brucellosis, for example, a marked degree of hypersensitivity is developed and a skin reaction, a slightly raised edematous area, appears in about six hours after the intradermal inoculation of preparations of soluble antigen of Brucella. The preparations have been given various names such as abortin (from *Br. abortus*), melitin (from *Br. melitensis*), brucellin and brucellergen. Johnin, a preparation of Johne's bacillus, *Mycobacterium paratuberculosis,* is used in the diagnosis of Johne's disease of cattle. Hypersensitivity occurs with regularity in glanders, and a skin reaction intermediate between the immediate and delayed types follows intradermal inoculation of a preparation of *Actinobacillus mallei* designated mallein. Similarly, in chancroid a hypersensitivity develops which is demonstrable as a skin reaction following intradermal inoculation of killed Ducrey's bacillus, and a hypersensitivity occurs in leprosy which results in a positive skin reaction to extracts of leprous tissue termed lepromin. Hypersensitivity to fungi is also not uncommon; the mycids or secondary sterile lesions which occur in some kinds of dermatophytosis are manifestations of hypersensitivity. Infection with *Coccidioides immitis* and *Histoplasma capsulatum* sensitizes, and a skin reaction to preparations designated coccidioidin and histoplasmin is demonstrable. Hypersensitivity also occurs in the virus disease, lymphogranuloma venereum, and a skin test, the Frei test, with mouse brain antigen or yolk sac culture of the virus, the latter marketed as Lygranum, has very considerable diagnostic value.

THE RELATIONSHIP OF HYPERSENSITIVITY AND IMMUNITY[1, 61]

The status of hypersensitivity with respect to effective immunity to infection has been of very considerable interest in relation to tuberculosis. It was early supposed by Koch that the development of tuberculin sensitivity was indicative of immunity, and its significance in this respect is indicated by the well-known *Koch phenomenon.* If tubercle bacilli are injected subcutaneously into a normal and a sensitized, *i.e.,* infected, guinea pig, the course of the subsequent infection is considerably different. In the normal animal the usual indurated nodule forms, which becomes necrotic to form a necrotic ulcer that persists while the infection spreads via the regional lymphatics, becomes generalized, and the animal dies. In the hypersensitive animal, however, an inflammatory reaction occurs, but there is no nodule formed; within a day or two the area becomes necrotic and finally sloughs without further spread, and the shallow ulcer heals rapidly. On the other hand, the inoculation of appropriate amounts of tuberculin markedly intensifies the cellular reaction about foci of infection, and the infection tends to spread rapidly. It would seem, therefore, that within limits the hypersensitivity is functional in effective immunity, but it may also be of very great disadvantage to the host. The whole question is of particular importance in relation to active immunization against tuberculosis.

As indicated earlier, hypersensitivity may take a variety of forms. Perhaps in large part because of clinical differences, there has been some tendency to regard the allergies as basically different, not only from anaphylaxis but from one another. It will be clear, however, that there are essentially complete analogies running through all the forms of hypersensitivity, and the differences are more apparent than real. They arise from various factors such as the portal of entry of the antigen and its effectiveness in stimulating an immunological response and the shock organ affected. Thus, there is no real difference between the atopic and nonatopic allergies that is not accounted for on the basis of the relative efficacy of the sensitizing antigen—a high degree of sensitivity is associated with ease of demonstration of

antibody and interferes seriously with desensitization since it is difficult to give enough antigen to produce an adequate desensitization. Such considerations as these are the basis of the generally accepted belief that hypersensitivity is basically the same regardless of its clinical manifestations.

It is also clear that hypersensitivity is basically an immunological phenomenon, involving the stimulation of antibody formation and the union of antigen and antibody. The paradoxical situation arising from the contrast, say, between the neutralization of toxin by antitoxin, and the acquirement of toxicity by a bland antigen, is more apparent than real and a consequence of emphasis. Bronfenbrenner[5] has illustrated the relationship by analogy with fire: the warmth, light, and other pleasing aspects of a grate fire are also present when the house burns down; both involve precisely the same mechanism, yet the emphasis on the consequences of the combustion is entirely different in the two cases.

REFERENCES

1. Banič, S.: Rapport entre l'hypersensibilité et l'immunité. Rev. d'Immunol., 1953, **17:**135–171.
2. Billingham, R. E., Brent, L., and Medawar, P. B.: Quantitative studies on tissue transplantation immunity. II. The origin, strength and duration of actively and adoptively acquired immunity. Proc. Roy. Soc., Ser. B, 1954, **143:** 58–80.
3. Boehme, D., and Dubos, R. J.: The effect of bacterial constituents on the resistance of mice to heterologous infection and on the activity of their reticulo-endothelial system. J. Exptl. Med., 1958, **107:**523–536.
4. Brambell, F. W. R., Brierley, J., Halliday, R., and Hemmings, W. A.: Transfer of passive immunity from mother to young. Lancet, 1954, **i:**964–965.
5. Bronfenbrenner, J.: Allergic state and its relation to hypersensitiveness and resistance. Amer. Rev. Tuberc., 1937, **36:**293–302.
6. Brown, G. C., and Kendrick, P. L.: Serologic response of infants to a multiple vaccine for simultaneous immunization against diphtheria, pertussis, tetanus, and poliomyelitis, in relation to the presence of specific maternal antibody. Amer. J. Pub. Health, 1960, **50:** 1529–1538.
7. Burnet, F. M.: Theories of immunity. Perspect. Biol. Med., 1960, **3:**447–458.
8. Burrows, W.: Effective immunity to enteric infections with special reference to cholera. Lectures on Theoretical and Applied Aspects of Modern Microbiology. University of Maryland, 1961–62.
9. Campbell, B., Sarwar, M., and Peterson, W. E.: Diathelic immunization—a maternal-offspring relationship involving milk antibodies. Science, 1957, **125:**932–933.
10. Campbell, D. H., and Garvey, J. S.: Factors involved in antibody formation. J. Infect. Dis., 1960, **107:**15–28.
11. Cannon, P. R.: The functional significance of specific agglutinins and precipitins. Physiol. Rev., 1940, **20:**89–114.
12. Cheng, H. F., et al.: Localization of antigens by autoradiography. Proc. Soc. Exptl. Biol. Med., 1961, **106:**93–97.
13. Dineen, J. K., and Perry, B. T.: Studies on the nature of antibody production during the in vitro culture of lymphoid tissues. Australian J. Exptl. Biol. Med. Sci., 1960, **38:**363–374.
14. Dixon, F. J., and Weigle, W. O.: The nature of the immunologic inadequacy of neonatal rabbits as revealed by cell transfer studies. J. Exptl. Med., 1957, **105:**75–83.
15. Dragstedt, C. A.: Anaphylaxis. Physiol. Rev., 1941, **21:**563–587.
16. Draper, L. R., and Sussdorf, D. H.: The serum hemolysin response in intact and splenectomized rabbits following immunization by various routes. J. Infect. Dis., 1957, **100:**147–161.
17. Edsall, G.: Immunization. Ann. Rev. Microbiol., 1955, **9:**347–368.
18. Eisle, C. W., McCullough, N. B., Beal, G. A., and Burrows, W.: Development of Brucella agglutinins in humans following vaccination for cholera. Proc. Soc. Exptl. Biol. Med., 1946, **61:**89–91.
19. Elberg, S. S.: Cellular immunity. Bact. Rev., 1960, **24:**67–95.
20. Fagraeus, A.: The plasma cellular reaction and its relation to the formation of antibodies in vitro. J. Immunol., 1948, **58:**1–13.
21. Fink, A. A.: Anaphylaxis in the mouse: Possible relation of the Schultz-Dale reaction to serotonin release. Proc. Soc. Exptl. Biol. Med., 1956, **92:**673–675.
22. Fishman, M.: Antibody formation in tissue culture. Nature, 1959, **183:**1200–1201.
23. Fox, J. P.: Live virus immunization. A consideration of basic principles and possible pitfalls. Arch. Environment. Health, 1961, **2:**490–498.
24. Geiger, W. B., and Alpers, H. S.: Mode of action of antigen and other smooth-muscle stimulants. Science, 1957, **125:**1141.
25. Gell, P. G. H., and Benacerraf, B.: Delayed hypersensitivity to simple protein antigens. Adv. Immunol., 1961, **1:**319–344.
26. Good, R. A., Bridges, R. A., and Condie, R. M.: Host-parasite relationships in patients with dysproteinemias. Bact. Rev., 1960, **24:**115–132.
27. Günther, O.: Antagonism and synergy of antigens. Bull. W.H.O., 1955, **13:**479–489.
28. Hale, J. H.: Duration of immunity in virus diseases. Adv. Immunol., 1961, **1:**263–282.
29. Halliday, R.: The absorption of antibodies from immune sera by the gut of the young rat. Proc. Roy. Soc., Ser. B, 1955, **143:**408–413.
30. Halliday, R.: Prenatal and postnatal transmission of passive immunity to young rats. Proc. Roy. Soc., Ser. B, 1955, **143:**427–430.

31. Harris, H.: Mobilization of defensive cells in inflammatory tissue. Bact. Rev., 1960, **24:** 3–15.
32. Harris, T. N., and Harris, S.: Cellular sources of antibody: A review of current literature. Ann. N. Y. Acad. Sci., 1960, **86:**948–965.
33. Holub, M., and Jaroskova, L. (eds.): Mechanisms of Antibody Formation. Academic Press, New York. 1961.
34. Hook, E. W., and Wagner, R. R.: The resistance-promoting activity of endotoxins and other microbial products. I. Their effect on *Salmonella typhosa* infections of mice. J. Immunol., 1959, **83:**302–309.
35. Hook, E. W., and Wagner, R. R.: The resistance-promoting activity of endotoxins and other microbial products. II. Protection against the neurotoxic action of influenza virus. J. Immunol., 1959, **83:**310–317.
36. Humphrey, J. H., and Jaques, R.: The release of histamine and 5-hydroxy-tryptamine (serotonin) from platelets by antigen-antibody reactions (in vitro). J. Physiol. 1955, **128:**9–27.
37. Laurent, L. J. M., and Parish, H. J.: Serum reactions and serum sensitivity tests. Brit. Med. J., 1952, **i:**1294–1297.
38. Leduc, E. H., Coons, A. H., and Connolly, J. M.: Studies on antibody production. II. The primary and secondary response in popliteal lymph node of the rabbit. J. Exptl. Med., 1955, **102:**61–72.
39. Lipton, M. M., and Steigman, A. J.: Neonatal immunity. I. Disparity in maternal-infant poliovirus antibody. Proc. Soc. Exptl. Biol. Med., 1957, **96:**348–352.
40. Maximow, A. A., and Bloom, W.: Textbook of Histology. W. B. Saunders Company, Philadelphia. 7th ed., 1957. *Also see:* Bloom, W., and Fawcett, D. W.: Textbook of Histology. Saunders, 8th ed., 1962.
41. McMaster, P. D.: Sites of antibody formation. In Pappenheimer, A. M., Jr. (ed.): The Nature and Significance of the Antibody Response, pp. 13–45. Columbia University Press, 1953.
42. Menkin, V.: Biochemical Mechanisms in Inflammation. Thomas, Springfield, Illinois. 1956.
43. Menkin, V.: Biology of inflammation; chemical mediators and cellular injury. Science, 1956, **123:**527–534.
44. Millican, R. C., Rust, J., and Rosenthal, S. M.: Gamma globulin factors protective against Pseudomonas and other organisms. Science, 1957, **126:**509–511.
45. Mitchinson, N. A.: Studies on the immunological response to foreign tumor transplants in the mouse. I. The role of lymph node cells in conferring immunity by adoptive transfer. J. Exptl. Med., 1955, **102:**157–178.
46. Neter, E.: Detection of incomplete enterobacterial antibodies in cord blood by means of antiglobulin hemagglutination test. Proc. Soc. Exptl. Biol. Med., 1957, **96:**488–491.
47. Nossal, G. J. V.: Antibody production by single cells. Brit. J. Exptl. Path., 1958, **39:**544–551.
48. Nossal, G. J. V.: Antibody production by single cells. II. The difference between primary and secondary response. Brit. J. Exptl. Path., 1959, **40:**118–124.
49. Nossal, G. J. V.: Antibody production by single cells. III. The histology of antibody production. Brit. J. Exptl. Path., 1959, **40:**301–311.
50. Nossal, G. J. V.: Antibody production by single cells. IV. Further studies on multiple immunized animals. Brit. J. Exptl. Path., 1960, **41:**89–96.
51. Nossal, G. J. V.: Studies on the transfer of antibody-producing capacity. II. The serial transfer of antibody-producing cells. Immunol., 1960, **3:**109–116.
52. Nossal, G. J. V., and Mäkelä, O.: Elaboration of antibodies by single cells. Ann. Rev. Microbiol., 1962, **16:**53–74.
53. Oakley, C. L.: Sites of production of antibodies. Proc. Roy. Soc. Med., 1953, **46:**1–4.
54. Ortega, L. G., and Mellors, R. C.: Cellular sites of formation of gamma globulin. J. Exptl. Med., 1957, **106:**627–640.
55. Ovary, Z., and Bier, O. G.: Quantitative studies on passive cutaneous anaphylaxis in the guinea pig and its relation to the Arthus phenomenon. J. Immunol., 1953, **71:**6–11.
56. Parrish, H. J., Barr, M., Ungar, J., and Butler, N. R.: Discussion of combined immunization. Proc. Roy. Soc. Med., 1954, **47:**351–360.
57. Pierce, A. E.: Specific antibodies at mucous surfaces. Vet. Rev. Annot., 1959, **5:**17–36.
58. Raffel, S.: The components of the tubercle bacillus responsible for the delayed type of "infectious" allergy. J. Infect. Dis., 1948, **82:** 267–293.
59. Raffel, S., and Forney, J. E.: The role of the "wax" of the tubercle bacillus in establishing delayed hypersensitivity. I. Hypersensitivity to a simple chemical substance, picryl chloride. J. Exptl. Med., 1948, **88:**485–501.
60. Raffel, S., and Newel, J. M.: The "delayed hypersensitivity" induced by antigen-antibody complexes. J. Exptl. Med., 1958, **108:**823–841.
61. Rich, A. R.: The significance of hypersensitivity in infection. Physiol. Rev., 1941, **21:**70–111.
62. Rogers, D. E.: Host mechanisms which act to remove bacteria from the blood stream. Bact. Rev., 1960, **24:**50–66.
63. Rosen, F. S.: The endotoxins of the gram-negative bacteria and host resistance. New England J. Med., 1961, **264:**919–923; 980–985.
64. Sabesin, S. M., and Banfield, W. G.: Electron microscopy of the Arthus reaction, using ferritin as antigen. Proc. Soc. Exptl. Biol. Med., 1961, **107:**546–550.
65. Speirs, R. S.: A theory of antibody formation involving eosinophils and reticuloendothelial cells. Nature, 1958, **181:**681–682.
66. Stavitsky, A. B.: In vitro studies of the antibody response. Adv. Immunol., 1961, **1:**211–262.
67. Stevens, K. M., and McKenna, J. M.: Studies on antibody synthesis initiated in vitro. J. Exptl. Med., 1958, **107:**537–559.
68. Sussdorf, D. H.: Rise of antibody titer in cell-free spleen preparations incubated in vitro. Nature, 1961, **191:**770–772.
69. Suter, E., and Hulliger, L.: Nonspecific and specific cellular reactions to infections. Ann. N. Y. Acad. Sci., 1960, **88:**1237–1245.
70. Symposium: Immunology and cancer. Ann. N. Y. Acad. Sci., 1957, **69:**525–856.
71. Symposium: Antibodies: Their production and mechanism of action. J. Cell. Comp. Physiol., 1957, **50:**Suppl. 1.
72. Symposium: Symposium on studies of antibody formation following cellular transfer. Fed. Proc., 1957, **16:**638–660.
73. Symposium: Symposium on tetanus. Proc. Staff Meetings Mayo Clinic, 1957, **32:**141–167.
74. Symposium: Proceedings of a symposium on

immunization in childhood. Livingstone, London. 1959.
75. Symposium: Connective tissue and diseases of connective tissue. Ann. N. Y. Acad. Sci., 1960, **86:**875–1132.
76. Symposium: The reticuloendothelial system (RES). Ann. N. Y. Acad Sci., 1960, **88:**1–280.
77. Symposium: Calandrier des vaccinations. Rev. d'Immunol., 1960, **24:**1–344.
78. Tokuda, S., and Weiser, R. S.: Production of anaphylaxis in the white mouse with soluble antigen-antibody complexes. Science, 1958, **127:**1237–1238.
79. Tully, J. G., and Gaines, S.: H antigen of *Salmonella typhosa.* J. Bact., 1961, **81:**924–932.
80. Weigle, W. O.: Fate and biological action of antigen-antibody complexes. Adv. Immunol., 1961, **1:**283–318.
81. Weigle, W. O., Cochrane, C. G., and Dixon, J. F.: Anaphylactogenic properties of soluble antigen-antibody complexes in the guinea pig and rabbit. J. Immunol., 1960, **85:**469–477.
82. Weissbach, H., Waalkes, T. P., and Undenfriend, S.: Presence of serotonin in lung and its implication in the anaphylactic reaction. Science, 1957, **125:**235–236.
83. White, R. G.: Antibody production by single cells. Nature, 1958, **182:**1383–1384.
84. Wood, W. B., Jr.: Studies on the mechanism of recovery in pneumococcal pneumonia. I. The action of type specific antibody upon the pulmonary lesion of experimental pneumonia. J. Exptl. Med., 1941, **73:**201–222.

Chapter 16

THE STAPHYLOCOCCI

THE STAPHYLOCOCCI are ubiquitous pathogenic microorganisms, are the commonest cause of localized suppurative infections, and are among the longest recognized of the pathogenic bacteria, having been characterized in the early 1880's largely through the work of Rosenbach. Nevertheless, they remain somewhat enigmatic and are less well known than the majority of bacteria.[24]

Morphology and Staining. The coccus form tends to be much more uniform in size than the other morphological types of bacteria, and the staphylococci are consistently slightly less than 1 μ in diameter, and typically are more nearly perfect spheres than many cocci. Their most obvious morphological characteristic is their marked tendency to occur as masses of cells. This is a consequence of cell division occurring in three planes, coupled with a tendency of the daughter cells to remain in close proximity to give the characteristic appearance. These irregular clusters are three-dimensional; this is apparent on examination of wet mounts, but in the usual stained smear preparation, the clusters are flattened out to give the appearance of irregular sheets of cells. While some cells may be found singly, in pairs, or even very short chains, this characteristic morphology serves to identify the staphylococci except under special circumstances, *e.g.*, it would be extremely difficult to differentiate staphylococci and many streptococci admixed in a smear.

These bacteria stain readily and deeply with the usual basic dyes in the simple staining procedure, and are strongly gram-positive, though rare gram-negative forms occur. They do not form spores; apparently do not have capsules except in the case of rare mucoid variants; and, although motile forms have been described, are almost invariably nonmotile.

Growth on agar mediums is abundant, and the colonies are opaque, smooth and glistening in appearance. Some staphylococci form lipochrome pigments which give the colonies a golden yellow or lemon yellow color, while others do not and are white. Although pigmentation is a variable characteristic (see below) and objected to as a differential characteristic by some,[12] it is constant in primary isolates and has assumed considerable practical significance. The golden pigmented forms are *Staphylococcus aureus* (*Staph. pyogenes* var. *aureus*, *Micrococcus pyogenes* var. *aureus*), which are predominant almost to the exclusion of other forms in pathologic processes. The lemon yellow pigmented forms are *Staph. citreus* and appear to be largely saprophytic. The white forms are known as *Staph. albus*, with an attempt to distinguish between the varieties found on the skin as a part of the normal flora as *Staph. epidermidis*, and *Staph. pyogenes* var. *albus* as the white staphylococci occasionally found in infectious processes; it is doubtful that such a distinction is valid or useful.

When cultured on blood agar plates,

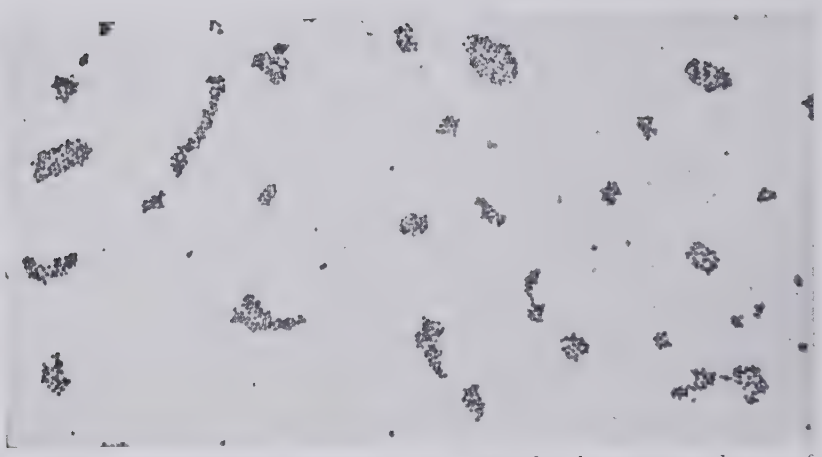


Figure 79. *Staphylococcus aureus* from pure culture. Note the characteristic clusters of the cocci. Fuchsin; × 1050.

some of the staphylococci are β-hemolytic, and others are nonhemolytic; green or α-hemolysis does not appear to occur among the staphylococci. There is an approximate association between hemolytic activity and the formation of golden pigment, and the typical pathogenic form is hemolytic *Staph. aureus*, although nonpigmented or nonhemolytic, or both, forms are occasionally found in apparently causal relation to pathologic processes. Conversely, golden pigmented forms are not found exclusively in association with disease; some saprophytic forms are similarly pigmented.

Physiology. The staphylococci are relatively more resistant to heat, and to a certain extent disinfectants, than the vegetative forms of most pathogenic bacteria. While most bacteria are killed in 30 minutes at 60° C., higher temperatures and longer times, as 80° C. for one hour, are often required for staphylococci. This assumes practical significance in the preparation of autogenous vaccines sometimes used in the therapy of persistent staphylococcal infections of the superficial tissues. They are also resistant to drying, and may remain infectious for extended periods, and are able to grow in the presence of relatively high, 10 per cent, concentrations of sodium chloride. The latter is significant in the preservation of foods with salt for staphylococci may grow and form enterotoxin (see below) in foods containing sufficient salt to act as a preservative otherwise. This property is also taken advantage of in the preparation of selective mediums for isolation of staphylococci.

As strongly gram-positive microorganisms, the staphylococci are sensitive to the bacteriostatic activity of triphenyl methane and other dyes, and are characteristically sensitive to those antibiotics effective on gram-positive bacteria, including penicillin and the broad-spectrum antibiotics such as the tetracyclines, but are quite insensitive to the antibiotics such as streptomycin whose antibacterial activity is confined to the gram-negative forms. They are especially prone to develop drug resistance (see below), and some of these generalizations are frequently not applicable to current isolates.

Selective mediums. The majority of the coagulase-positive (see below) pathogenic staphylococci are able to grow in the presence of tellurite, reducing it to give grey-black colonies on tellurite-containing mediums. In throat cultures on such mediums they may be confused with diphtheria bacilli which are also resistant to tellurite and able to reduce it (Chap. 30). Tellurite mediums tend to be selective and have been useful in the isolation of staphylococci from heavily contaminated material such as fecal specimens.[94] These staphylococci also often produce a zone of opacity in the medium around the colony when it

contains egg yolk, and this characteristic has been combined with tellurite reduction in the development of selective isolation mediums.[43] Mannitol (see below) and an acid-base indicator such as phenol red may be incorporated for differential, though not selective, purposes; and isolation mediums may be made selective by the inclusion of 10 per cent sodium chloride.[57]

Nutritive requirements. The staphylococci are not highly fastidious in their nutritive requirements, and grow readily on the usual meat extract-peptone mediums, but they grow more profusely on blood agar commonly used for isolation of the pathogenic forms. On semi-synthetic mediums containing casein hydrolysate, nicotinic acid and thiamin are commonly required, and growth may be enhanced by the inclusion of additional bacterial vitamins, as biotin, in these and simpler mediums. In chemically defined mediums, freshly isolated strains require a considerable number of amino acids, usually cystine, leucine, proline, valine, glycine, aspartic acid, phenylalanine and arginine, but the precise requirements differ from strain to strain. The bacteria can be "trained" by passage on successively simpler mediums to grow in the absence of most, or all, of the amino acids required by the parent strain.[34] In general, freshly isolated strains of *Staph. aureus* are the most exacting in their nutritive requirements, and old laboratory cultures and *Staph. albus* less so.

Cultural characteristics. Lactic acid is the predominating end-product of glucose fermentation, and small amounts of ethanol and carbon dioxide are formed. The fermentation of the conventional sugars and polyhydric alcohols is irregular and, for the most part, has no differential significance. Lactose, sucrose, maltose, glycerol, mannose, fructose and erythritol are usually fermented; the fermentation of raffinose, inulin and salicin is variable; and inositol, dulcitol, L-xylose, rhamnose, L-arabinose, adonitol, D-sorbitol, cellobiose and dextrin are not fermented, and starch is not hydrolyzed. The fermentation of mannitol is considered to have differential significance in that it is fermented by the majority of coagulase-positive strains. Of the other conventional biochemical reactions, indol is not produced from tryptophane, gelatin liquefaction is variable as is the reduction of nitrate. In sum, the biochemical reactions of the staphylococci, with the possible exception of the mannitol fermentation, do not provide a basis for their differentiation.

Antigenic Structure.[65] The antigenic structure of the staphylococci of human origin has been studied in various ways, for the most part by agglutination, but also as the serological activity of soluble extracts in precipitin, gel diffusion and passive hemagglutination reactions. Agglutination studies are complicated by the effects of blocking antigens, but these are minimized by using very young, four to six hour, cultures. Appli-

Figure 80. Colonies of *Staphylococcus aureus* on nutrient agar. Twenty-four-hour culture; × 3.

cation of the conventional antigenic analysis technique has shown the presence of 13 antigens, arbitrarily designated by lower case letters, some heat-stable and some heat-labile. Typing is relatively difficult, and the slide agglutination test is considered to be much more reliable than tube titration.

Soluble antigens, apparently largely carbohydrate in nature, prepared by extraction in hot dilute acid, allow the separation of the staphylococci into two groups, designated A and B; type A strains were originally thought to be pathogenic and type B nonpathogenic. It subsequently became clear that this differentiation is not clear-cut and these antigens do not occur in all strains. A protein antigen was prepared by Verwey which was strain- and type-specific, but its general distribution among the staphylococci is not known. Other types of soluble antigens, nucleoprotein in nature, have been prepared which show cross reactions with streptococci and pneumococci, and it is probable that these coccus forms share some antigenic components.

Attempts to correlate antigenic structure with other characteristics have not been successful. A correlation with phage type (see below) is believed by some[69] to occur, but not by others.[66] There seems to be no correlation with virulence and serological methods do not contribute to definition of pathogenic strains.[39]

Phage Typing.[7, 9] The host specificity of the bacterial viruses described elsewhere (Chap. 4) provides an additional means of characterization of bacteria, extending to strain differences. This method of differentiation was first applied to the typhoid bacillus (Chap. 21), allowing characterization within a single serotype, and has been extremely useful for epidemiological purposes. The application of bacteriophage typing, or phage typing, to the staphylococci has been largely the work of Blair and has been invaluable in providing a means of identification where the conventional biochemical and serological methods have thus far failed.

In brief, phage typing is based upon a collection of a group of phages of varied specificity but which in the aggregate will lyse the great majority of strains of bacteria encountered; these phages are numbered arbitrarily. While the maintenance of such standard typing phages requires technical precision, and test conditions must be defined, the typing procedure is simple. It is carried out by spotting the phages in appropriate dilution, and usually in two concentrations, on a petri dish culture of the bacterium inoculated to give a uniform film of growth. Sensitivity to a phage is indicated by the clear zones of confluent lysis, grossly similar to plaques but not clonal in nature. When the bacterium is not infected by the test phage the film of growth is uniform.

Strain-specific phages are found but are rare, and phage typing is based upon a kind of spectrum or pattern of activity which is defined for the standard phages against standard test strains of bacteria. The number of such patterns is very large, impractically so, and it is recommended that for routine work a set of at least 21 basic phages should be used and grouped as follows:

Group I: 29, 52, 52A, 79, 80
Group II: 3A, 3B, 3C, 55, 71
Group III: 6, 7, 42E, 47, 53, 54, 75, 77
Group IV: 42D
Miscellaneous: 81, 187

It is thus possible to characterize a strain of staphylococcus as belonging to one or another of these groups and phage types.

Such phage typing appears to be arbitrary in the present state of knowledge, *i.e.*, the nature of the susceptibility of a bacterium to infection with a bacterial virus is not yet subject to precise definition. Among the enteric bacilli, the phage receptor on the bacterial cell may be related to the O antigen complex but, as noted above, a similar relation of contained antigen of staphylococci is quite uncertain.

Toxins.[6, 13, 54, 56] It was apparent very early that cell-free filtrates of cultures of staphylococci are toxic on parenteral inoculation, and that extra-

cellular toxin(s) is formed in considerable amount. The more obvious effects in experimental animals, commonly the rabbit, have been attributed to a necrotic, or dermonecrotic, toxin (dermonecrotoxin) and to a lethal toxin. In the case of the former, intradermal inoculation produces an intense inflammatory reaction, the center of the lesion becomes edematous, and then necrotic about three days after inoculation. The prominent histologic feature is necrosis of the vessels, and the lesion so produced heals slowly. This activity appears to be closely similar among strains of staphylococci as indicated by protection by heterologous antiserums.

The lethal effect of the toxicity on the rabbit following intravenous inoculation may be a dramatic one. The incubation period is proportional to the dose, and may range from as little as two minutes to as long as 24 hours with very small doses. The toxicity affects the heart directly and also the vascular supply to the lungs to give acute failure of the right heart which is the immediate cause of death. On intracerebral or intrathecal inoculation, the toxicity acts upon the nerve cells, and death occurs very rapidly as a consequence of respiratory failure.

These toxicities, and the activities of the α-hemolysin and Neisser-Wechsberg leucocidin (see below) disappear on treatment with formaldehyde to give a toxoid. There is a prevalent belief that these several toxicities represent the activity of a single substance, but there are discrepancies in the evidence,[68, 72, 73] and this is not fully established.

In addition to these, there are a number of other toxic activities. Among the more important are the staphylococcal hemolysins or staphylolysins, the enterotoxin, the leucocidins, and the enzymatic or kinase activities coagulase, hyaluronidase, and fibrinolysin or staphylokinase. Still other toxicities may be observed occasionally; for example, the formation of a scarlatinal or scarlatinal-like toxin has been observed, and gives a scarlet fever of staphylococcal etiology.[18, 64, 79]

Staphylolysins. The pyogenic staphylococci are almost invariably hemolytic, and on primary isolation on blood agar the colonies are surrounded by a clear zone of β-hemolysis as described above. The hemolytic activity is also present in cell-free filtrates of cultures, and consists of several distinct hemolysins, or staphylolysins.

The first to be differentiated were the α- and β-lysins. These differ immunologically, in the species of red cells lysed, and in certain other characteristics. The α-lysin lyses sheep and rabbit red cells, but not human, guinea pig or horse cells. It requires magnesium or manganese ions and is inhibited by chelating agents. The β-lysin acts on sheep but not rabbit cells, and has only slight hemolytic activity on human cells. It is produced by staphylococci of animal origin for the most part and is uncommon in human strains. It is also distinguished from the α-lysin in that it is a hot-cold lysin, *i.e.*, except in very high concentrations, it does not lyse red cells when incubated at 37° C., but on chilling after such incubation the cells are lysed. It also requires magnesium ions and the activity which disappears on treatment with chelating agents is restored by their addition.[49] In the warm phase incubation, the β-lysin potentiates the lysis of sheep cells by certain other hemolysins: that of group B streptococci (*Str. agalactiae*) and the δ-staphylolysin. A third hemolysin, the δ-lysin, differs immunologically from the α- and β-lysins, and, unlike them, lyses red cells from man, monkey, horse, rat, mouse and guinea pig, as well as sheep and rabbit cells.[45, 47, 48] It differs also in that it reaches a peak in titer in 48 hour cultures rather than after 96 hours incubation as in the case of the α- and β-lysins. Two other hemolysins have been described: the γ-lysin which closely resembles the α-lysin but differs immunologically and whose existence has been disputed[24]; and the ϵ-lysin which is reported to occur exclusively in nonpathogenic staphylococci.

These hemolytic activities may be associated with other manifestations of toxicity. The evidence suggests that the

α-lysin is also a leucocidin (see below), and it is closely associated with the dermonecrotic activity. The δ-lysin has also been found to be dermonecrotic and lethal in purified preparations. The β-lysin shows some toxicity on intravenous inoculation of rabbits, but is much less toxic than the α- and δ-lysins.

Of these staphylolysins, the α- and δ-lysins predominate in strains producing disease in man and are highly correlated with the formation of coagulase (see below), more than 95 per cent of such coagulase-positive strains forming one or the other, and 82 per cent forming both. There is a similar high incidence of these lysins in coagulase-positive strains found on the skin; coagulase-negative strains from the same source do not form the α- and δ-lysins, but 95 per cent form ε-lysin. In contrast, α- and δ-lysins are less common in pathogenic strains of animal origin, occurring together in the absence of other lysins in only 10 per cent of strains, but the β-lysin is found in 88 per cent of such strains, and in combination with the α- and δ-lysins in 59 per cent. The incidence of these hemolysins is summarized in some detail by Elek and Levy.[25]

Leucocidins.[35] The toxic effect of staphylococcal products on heterophils was observed prior to the turn of the century. Assay of this activity was put on a quantitative basis by the bioscopic method of Neisser and Wechsberg, using rabbit cells and measuring the toxic effect as the inability of the cells to reduce intracellular methylene blue, *i.e.*, an inhibition of respiration. Heterophils from man, mouse and guinea pig are relatively resistant to this toxicity, and those from the frog almost completely resistant.

Human heterophils were used by Panton and Valentine[68] and the staphylococcal activity was found to inhibit phagocytosis. The Neisser-Wechsberg leucocidin is apparently identical with the α-lysin, and for all practical purposes affects only rabbit cells. In contrast, the Panton-Valentine (P-V) leucocidin, active on human as well as rabbit cells, is also antigenically distinct, and is formed by strains of staphylococcus which do not form α-lysin as well as those which do, *i.e.*, it occurs independently. It reaches peak titer within 24 hours incubation, and is oxygen-labile and declines in titer as incubation is continued.

These two leucocidins differ in their effects on heterophils. The α-lysin produces an agglutination of leucocytes and kills them as indicated by their staining with methylene blue, but the cells are not lysed. The P-V leucocidin produces a swelling of the cells to a spherical shape, with granules arranged around the periphery of the cell, and eventually the cell bursts and the granules are liberated.

A third leucocidin, designated leucolysin,[35] has been described which differs from the P-V leucocidin in that it is thermostable, is active on the heterophils of all species tested except sheep, and is inhibited by cholesterol. It produces a nuclear swelling in the affected heterophils which are lysed in high concentrations of the activity. It is closely associated, if not identical, with the δ-lysin whose leucocidin activity had been described earlier.[46]

Enterotoxin.[16] The relation of staphylococci to the intoxication type of food poisoning described elsewhere (Chap. 12) was indicated by a number of observations, notably that of Barber in 1914 on acute gastrointestinal upsets associated with drinking milk from a cow with staphylococcal mastitis. It was not until 1930 that it was shown by Dack and his associates that such illness is produced by ingestion of cell-free culture of staphylococus strains which form enterotoxin.

This activity is completely unrelated to other toxicities of staphylococci. It appears to be formed only by coagulase-positive staphylococci, but not by all such strains. Enterotoxic strains are most commonly, 81 per cent of those typed, of phage types 6/47 or 42D,[1] but not all strains of these phage types are enterotoxic; the former is one of the

commonest types carried by nasal carriers, and the latter a frequent cause of bovine mastitis. There appears to be no relation between the conventional biochemical properties of staphylococci and the production of enterotoxin.[27] At one time gelatinase production and growth in the presence of excess sodium chloride were thought to be associated with enterotoxin production, but this is not true. In short, there is not as yet any method of identifying enterotoxic strains of staphylococci other than by demonstration of toxin formation.

Susceptibility to enterotoxin appears to be limited to man and certain monkeys. In man an acute gastrointestinal upset follows ingestion of the toxin in two to three hours, and is characterized by projectile vomiting and diarrhea. Symptoms subside after a few hours, and recovery is complete by the following day. In adults the disease is practically never fatal, but fatal toxemia may occur in infants, and is presumably related to lack of gastric acidity. Man is apparently much more susceptible to the enterotoxin than various monkeys, two to five ml. of cell-free culture filtrate usually sufficing to produce illness, but occasional individuals are resistant. Much of the early experimental work was carried out with human volunteers.

A number of New World monkeys are susceptible and develop diarrhea and malaise, though usually not vomiting, following intragastric inoculation. The macacus monkey, *Macaca mulatta*, is the commonly used experimental animal. When a fairly large dose, 50 ml., of staphylococcus culture filtrate is given intragastrically, most, though not all, animals respond with diarrhea and vomiting after an incubation period of two to three hours, and symptoms subside a short time later. All monkeys do not respond, and susceptible animals may fail to respond after several, or even a single, such inoculation. Chimpanzees have been reported to be much more susceptible.[91] Earlier reports that kittens respond specifically to enterotoxin by the oral route with vomiting have not been substantiated, and the kitten test, which unfortunately has been widely applied, is not considered to be reliable.[1] The lack of a susceptible small laboratory animal is a severe handicap in the study of enterotoxin.

The nature of the toxic action is not established. One view is that the toxin is a neurotoxin which is absorbed from the bowel and vomiting results from its action on the vomiting center or the peripheral nerve structures, or both. The production of characteristic symptoms in the monkey by intravenous inoculation of enterotoxin is consistent with this, but secretion into the gut is not ruled out. The enterospasm has been considered by others as indicating a direct action on smooth muscle.[2, 67]

The enterotoxin appears to be protein-like in nature and may be separated from toxic filtrates by the conventional salting out and ethanol and methanol precipitations. It is relatively resistant to heat, withstanding boiling for 30 minutes at neutrality, but is partially destroyed in an hour, and completely destroyed on autoclaving. Evidence from both *in vivo* and *in vitro* studies as to the antigenicity of enterotoxin is conflicting. There is no doubt that a tolerance develops, sometimes very rapidly, in monkeys, and possibly also in man, but whether it rests upon an antibody basis is another matter. If immunization is effective, as indicated in some studies, the protective effect is of a very low order. Serological studies have given conflicting results, and as yet there is no readily applicable serological test for the identification of enterotoxin-producing strains of staphylococci; gel diffusion methods have been the most promising, and suggest the possibility of more than one antigenic type of enterotoxin.[14, 82, 83, 84]

Coagulase. The ability of some bacteria, and staphylococci in particular, to clot plasma has been described elsewhere (Chap. 9). Staphylocoagulase has been of special interest because of its high correlation with virulence of staphylococcus strains of human and animal

origin, and because of effects, such as protection of the bacteria against phagocytosis, antagonism of normal bactericidal activity of serum, etc., which plausibly relate to microbial virulence.

The activity is independent of the other staphylococcal toxins although associated with activities such as the α- and δ-lysins which also characterize virulent strains. It may be obtained cell-free although the results are somewhat erratic; the inconsistencies have been accounted for by the occurrence of bound and unbound coagulase,[20, 70] but the existence of two such forms is questioned by some.[44] Cell-free coagulase[21, 28, 58] may be purified by salting out and ethanol fractionation.[11, 86] It is apparently protein in nature, containing little or no carbohydrate, and is readily inactivated by proteolytic enzymes. It is relatively heat resistant, to 60° C. for 30 minutes, and is only partially inactivated at 100° C. in the same time. It is antigenic and serological studies[19] have shown that at least four antigenic types, designated A, B, C and D, are demonstrable.

Coagulase activity *in vitro* is usually assayed in one or the other of two ways. One consists in mixing the bacterial culture with plasma, human or rabbit, and observing clotting. Calcium is not a factor and citrated or oxalated plasma may be used. This may be made more precise by using purified fibrinogen together with serum activator. For qualitative, and routine, purposes the plasma clumping test is used; the bacteria are suspended in a drop of plasma and coagulase-positive strains clump rapidly, giving the appearance of a slide agglutination test. The deposition of fibrin on the surface of the cells results in clumping, but the test does not precisely parallel the tube test. The presence of 1:10,000 Merthiolate, or inactivation of the plasma, interfere with the tube test but not the slide test. It has been suggested that the tube test measures free coagulase, and the plasma clumping test free or bound coagulase.[20, 70]

The bactericidal activity of normal serum toward staphylococci is markedly inhibited by coagulase,[22, 23] but the mechanism of the effect is uncertain; it is apparently not concerned with the respiratory metabolism of the bacteria.[93] Coagulase is not highly toxic on parenteral inoculation, but in sufficient doses produces a rapid fall in fibrinogen and extensive intravenous clotting, especially in the lungs, to produce rapid death.[10, 80]

Hyaluronidase.[55, 74, 77] This enzymatic activity, which depolymerizes the ground substance of the tissues, hyaluronic acid (Chap. 9), is formed by the majority of pathogenic staphylococci and is thus associated with other characteristics such as α- and δ-lysin formation, coagulase production, etc. Its enzymatic activity appears to be the same as that of hyaluronidases from other sources. While hyaluronidases of different origin are serologically different, even between groups of streptococci, staphylococcal hyaluronidase appears to be antigenically homogeneous.

Staphylokinase.[54] Fibrinolytic activity has been studied more intensively with streptococci than with staphylococci, but a considerable portion of staphylococci isolated from human carriers and from diseased tissue will dissolve fibrin clots. The mechanism of staphylococcal fibrinolysis is similar to that of streptococcal fibrinolysis described earlier (Chap. 9) in that the bacterial product is a kinase which activates a serum or plasma protease to give the lytic activity. It differs from streptokinase in that it acts on the plasma of animal species other than man, including the dog, guinea pig, and rabbit, but not the cow, and requires a longer incubation time. It is readily differentiable from staphylocoagulase in that it is heat-labile. A curious observation reported by a number of workers is that strains of staphylococci that produce β-lysin do not produce staphylokinase.

Variation. Like other bacteria the staphylococci undergo S-R dissociation. These variants have not been thoroughly studied with respect to the various toxins described above, and available information is limited. The characteristics

used to differentiate the staphylococci, *viz.*, pigmentation, toxigenicity in the broad sense, and biochemical activity, are all subject to variation, and are considered in connection with classification (see below).

Drug resistance. The staphylococcal variation of by far the greatest practical importance is that in susceptibility to the antimicrobial activity of the antibiotics, and has been considered elsewhere (Chap. 7) as an outstanding example of acquired resistance. The staphylococci appear to become drug-resistant more readily than most other bacteria. They may be made resistant to antibiotics by culture in the presence of successively increasing concentrations of these substances, and they become drug-resistant under natural conditions. The appearance of drug resistance in strains isolated from pathologic processes has followed the introduction of the various antibiotics into general use, and the proportion of resistant strains found has continuously increased.

Penicillin was the first antibiotic to be used, and penicillin resistance appeared shortly after its introduction, followed by resistance to the tetracyclines, erythromycin, etc. Chloramphenicol resistance occurs also, but to a lesser extent, probably because this antibiotic has been used less frequently as a consequence of its association with damage to hemopoietic tissue and the occurrence of aplastic anemia following its continued administration. Drug resistance has taken the form of multiple resistance of individual strains of staphylococci, and strains resistant to more than one antibiotic are by now the rule rather than the exception. In fact, the incidence of drug-resistant staphylococci has reached a point at which the status of treatment and control of staphylococcal infections has, in the opinion of many, essentially reverted to that of the pre-drug era. This is of particular significance in view of the increasing prevalence and fatality of these infections.

Staphylococcal infections tend to be acquired in hospitals, and the proportion of drug-resistant strains found in carriers is much higher among hospital personnel than in the general population.[30, 85] Precise figures vary from one area to another, and change with time in a single hospital, but in general 65 to 90 per cent of strains are penicillin-resistant, perhaps 50 per cent tetracycline-resistant, 20 per cent erythromycin-resistant, etc., in hospitals, while the proportions are much lower, possibly 5 to 15 per cent penicillin-resistant, in the general population.

Extensive use has been made of phage typing in efforts to trace the sources of infection acquired in hospitals. The phage type 80/81 appears to be the most often penicillin-resistant, but this phage type is also a prevalent virulent one (see below). Retrospective studies, by subsequent determination of phage type of earlier isolates, of strains isolated over the years 1927 through 1947[8] have shown that the 80/81 type (described in 1955) was prevalent in this period, but that there was a relatively low incidence of phage types of group III, which increased after the introduction of antibiotics. Subsequently strains of group I increased, largely at the expense of group III.[31, 89] The significance of such observations is not clear, but they no doubt reflect to a degree the frequency of phage types of virulent staphylococci (see below).

Experimentally, and apparently also under natural conditions, resistant strains of staphylococci tend to revert to sensitivity, illustrated in the latter instance as a reduced frequency of resistant isolates. This tendency makes possible an effective approach to the problems posed by the occurrence of resistant strains, *i.e.*, through control of the kinds of antibiotics used for therapeutic purposes. That such control may have practical consequences is illustrated in an experience with hospital populations in which therapeutic efficacy was improved by use of antibiotics indicated by the frequency of isolation of resistant strains.[3]

Penicillin resistance among the staphylococci is of two kinds. That induced by laboratory procedures is usually accom-

panied by morphological changes, notably the loss of gram-positiveness, and is associated with alterations in glutamic acid metabolism described earlier (Chap. 7), and is of a temporary character in that reversion occurs with some facility. Penicillin-fast staphylococci isolated from infection, however, usually show no morphological changes, and produce penicillinase. The significance of penicillinase production, *i.e.*, resistance by virtue of decomposing the antimicrobial agent, is underscored by the sensitivity of penicillin-resistant strains of staphylococci to 2,6-dimethoxyphenyl penicillin (Staphcillin, Celbenin, Methicillin, BRL 1241), which is not degraded by penicillinase to the inactive compound penicillinoic acid.[29, 53, 76,87] Resistant strains may be able to decompose penicillin by other enzymes; penicillin acylase, which decomposes penicillin to the relatively inactive 6-aminopenicillanic acid, has also been described as responsible for penicillin-resistance.[26]

Differentiation. Differentiation of species or types of staphylococci has centered largely around the definition of virulence of these bacteria in terms of associated characteristics. Virulence is assessed in two ways: the source of the strain of bacteria, *i.e.*, whether or not from a disease process, usually suppurative, in which it appears to be the primary etiological agent; and pathogenicity for experimental animals (see below). *Staph. aureus* and *Staph. albus* appear to constitute the extremes in a continuous series of types, ranging from the virulent, pigmented, toxigenic, biochemically active forms, to the feebly pathogenic, almost saprophytic, nonpigmented biochemically inactive varieties.

The correlation between coagulase activity and virulence is very high, and the terms virulent and coagulase-positive have come to be almost interchangeable. Coagulase-positive staphylococci are almost invariably pigmented, *e.g.*, *Staph. aureus*, usually ferment mannitol under both aerobic and anaerobic conditions, and dextrose, maltose, and glycerol, and liquefy gelatin. The mannitol fermentation is considered by some to have differential significance because it is highly correlated with coagulase; it has been reported that about 1 per cent of coagulase-positive strains are mannitol-negative, and about 8 per cent of mannitol-positive strains are coagulase-negative.[52] Coagulase-positive strains are practically invariably hemolytic on blood agar, and there appears to be a high correlation between the formation of α- and δ-lysins and coagulase. In one study, of 532 coagulase-positive strains isolated from carriers, all α-lysin-positive strains were also coagulase-positive, and only 4 per cent of coagulase-positive strains were α-lysin-negative, and all of 100 strains isolated from suppurative infections in man were α-lysin-positive. Of 200 coagulase-positive strains isolated from man and assayed by the gel diffusion method, 82 per cent produced both α- and δ-lysin, 11 per cent produced α-, β-, and δ-lysin, 3 per cent α-lysin only, and 4 per cent δ-lysin only. In total, then, 96 per cent produced α-lysin, and 97 per cent δ-lysin. It will be recalled that the α-lysin is also a leucocidin, and the δ-lysin is both dermonecrotic and lethal. In contrast, of 77 coagulase-negative strains, none produced α-, β-, or δ-lysins, but 95 per cent produced ϵ-lysin, and the remainder were not hemolytic. On the basis of information such as the foregoing, then, it is possible to define tentatively a virulent staphylococcus as one that is coagulase-positive and produces α- and/or δ-lysins.

Classification. The formal classification of the staphylococci is, like that of many other bacteria, not completely satisfactory. Most workers accept three general groups of these organisms, *viz.*, the *Staph. aureus-Staph. albus* series of strains or types with species status of the two extremes, virulent *Staph. aureus* and the relatively avirulent *Staph. albus* of the normal flora of the human skin, mouth, and upper respiratory tract; a group of saprophytic staphylococci found in air, milk, etc., and including the lemon-yellow pigmented *Staph. citreus*, varieties forming pink to reddish brown pigments, and colorless forms closely resembling *Staph. albus;* and,

finally, a group of obligate anaerobic staphylococci which are among the normal flora of the human body cavities, and one of which, *Staph. aerogenes*, is occasionally a cause of puerperal sepsis.

Pathogenicity.[4] Except in the case of staphylococcal pneumonias, these microorganisms enter the body either through the intact skin or following breaking of this barrier by trauma. The route of infection into the intact skin appears to be the hair follicles or sweat ducts. The staphylococcal infections commonly assume a localized form with a focus of purulent infection partially or completely walled off from surrounding tissues. This may be limited or may spread via the blood stream to give rise to secondary foci of infection in whatever tissues or organs the bacteria may lodge. Occasionally the infection may assume a fulminating bacteremic form.

The character of these infections is attributable, at least in part, to the properties of the toxic products of the microorganism. The close association of virulence with the formation of coagulase and the α- and δ-lysins allows a partial explanation of the pathogenesis of the infections produced. It seems probable that the tendency of the focus of infection to be walled off can be attributed to the action of coagulase, and the purulent character of the lesion to the necrotic activity and killing of locally mobilized leucocytes by the α- and δ-lysins.[42] The deposition of fibrin on the surface of the bacteria appears to interfere with phagocytosis, and even after phagocytosis, coagulase-positive staphylococci tend to persist in viable form.[15, 61] The development of metastatic abscesses or focal infections, as in the bone giving rise to staphylococcal osteomyelitis, is a consequence of hematogenous spread of the infection in the form of thrombi and phagocytic cells containing viable microorganisms.

Pathogenicity for man.[60, 75] Staphylococci are constantly present on the skin and in the upper respiratory tract. These are commonly the relatively avirulent *Staph. albus*, but virulent *Staph. aureus* occurs, especially in the upper respiratory tract.

Carriers. Nasal carriers are probably the most important single reservoir of staphylococcal infection of man. The increasing significance of infection with antibiotic-resistant staphylococci has resulted in a resurgence of interest in the carrier and the control of carrier-disseminated infection.[59] The spread of infection, airborne or by contact, is of particular concern in surgical wards[78] and in maternity wards.[33] The former is a prolific source of staphylococcal wound infection, and in the latter the newborn become infected within the first few days of life, and show a rate of perhaps 90 per cent on discharge. Examination of available data for the Scandanavian and Commonwealth countries and the United States over the period 1937–1959[62] has shown wide fluctuation in the carrier rates, from 20 to 60 per cent approximately, a reflection of the entry of penicillin into general use in the 1940's, and no general tendency to reduction, *e.g.*, rates of 40 to 50 per cent are recorded for 1959.

Suppurative Infections. A transitory drop in resistance may be sufficient to allow local invasion and the establishing of a focus of infection, perhaps acne or a simple boil, pemphigus or impetigo neonatorum, or a more or less extensive carbuncular condition may develop possibly followed by the appearance of metastatic abscesses or bacteremia. Whether a focus of infection is initially established following penetration of the mechanical barrier, and the course of events following its establishment, are functions of the balance between the virulence of the microorganism and the natural immunity of the host. Thus a variety of lesions and diseases of the skin are of staphylococcal etiology, as are a large majority of cases of osteomyelitis and periostitis, many cases of otitis media and sinusitis and, much less often, urinary tract infections and the relatively rare staphylococcal meningitis. In general, suppurative inflammation, in whatever part of the body it may occur, is usually associated with the presence of staphylococci either in pure or mixed culture. When they occur in

mixed infections, staphylococci may represent either primary or secondary invaders; it is often impossible to determine the sequence of events.

Enteritis.[17, 40, 90] Staphylococcal enteritis came into increasing prominence with the general use of antibiotics, especially the broad-spectrum antibiotics given by mouth. Such therapy, or prophylaxis, favors the growth of resistant staphylococci in the bowel at the expense of the normal flora; staphylococci occur frequently in the bowel and are readily detectable in 10 to 15 per cent of persons with no enteric disease. The disease occurs more commonly in surgical patients given prophylactic antibiotic therapy. The severity of the enteritis is approximately related to the relative numbers of staphylococci present, and in severe cases the intestinal flora may be grossly abnormal, almost completely lacking in gram-negative bacteria and consisting largely of enormous numbers of staphylococci admixed with pus cells as observed in fecal smears. On autopsy, the enteritis is usually found to be a pseudomembranous type. It is not clear what part staphylococcal toxins, such as enterotoxin and the staphylolysins, play in the pathogenesis of the disease, or the role, if any, of staphylococci in ulcerative colitis.

Pneumonia.[32, 38, 81] Staphylococcal pneumonias are not common, and are probably almost always secondary, often to influenza[71]; the alveolar edema of influenza is thought to favor staphylococcal infection. Two general types are recognized. One is a gradually developing cavitating pneumonia in which the initial lesion is a diffuse pulmonary mottling which coalesces to give consolidation and cavity formation. The other is a fulminating, hemorrhagic pneumonia with profound toxemia and is often accompanied by bacteremia. This second type is often secondary to influenza, and occurs in infants.[92] The bacteria may be found, frequently in almost pure culture, in sputum, empyema fluid, or lung puncture specimens, and in the blood when there is bacteremia. Intratracheal or intrabronchial inoculation of rabbits with cell-free filtrates has been found to result in consolidation, peribronchial areas of hemorrhagic congestion and necrosis, and blood vessel damage leading to ischemia of large areas of lung, a picture considered similar to that found in early cases of staphylococcal pneumonias in man.[50]

Prevalence. Staphylococcal infections have increased in prevalance and fatality, practically displacing the pneumococcus as the immediate etiological agent in terminal infection, and these infections, together with tuberculosis and enteric infections, have become the predominant bacterial infections in this country. Epidemiological studies have shown that there is a marked association between particular phage types and virulence as inferred from the source of the strain.[5] A large portion of virulent strains, to as much as 40 to 90 per cent in a given hospital, belong to the 80/81 complex. This complex includes strains which are also lysed by 52 and 52A phages, and the complex may be taken to include 80, 81, 80/81, 80/81/52/52A, and 80/52/52A.[63] There is no evidence that the staphylococci have become more virulent; in fact, primary hematogenous osteomyelitis has declined in incidence. It is more probable that these infections have increased at the expense of others more susceptible to chemotherapy, and it is possible also that factors such as skin punctures for diagnostic purposes and the more general use of x-ray and cortisone may have contributed to a reduced resistance to infection with these microorganisms.

Food Poisoning.[16] Food poisoning of staphylococcal etiology is quite common; it makes up the great majority of outbreaks of food poisoning in this country, and it is probable that there are few adults who have not experienced one or more attacks. It may be emphasized that this disease is not an infection, but an intoxication resulting from the ingestion of preformed enterotoxin. The sequence of events leading up to this kind of food poisoning, then, must include a period of incubation intervening between the preparation of the food and its ultimate

consumption. The enterotoxigenic staphylococci are commonly virulent *Staph. aureus* and are derived originally from man.

The contamination of the food may be direct, as by an infected handler, or it may be indirect in that one of the ingredients, such as bulk eggs, may be infected. The kinds of foods most frequently causing food poisoning of this kind are of such a nature that the initial contamination is possible, and their preparation includes a period of incubation to allow proliferation of the staphylococci with coincident formation of enterotoxin. Such foods are often bakery goods with soft fillings such as cream puffs, cream pies, or cake fillings, or gravies and some meats such as ham that are not prepared immediately prior to consumption. For example, cream puffs prepared and filled in the late afternoon or evening are left on the shelf in a warm room in the bakery overnight and sold for consumption the following day. Or the chicken gravy is removed from the refrigerator and allowed to stand at the temperature of a warm summer's day for some hours prior to consumption at a picnic. There is no overt evidence of the multiplication of the contained staphylococci, *i.e.*, obvious spoilage does not result, and the preformed enterotoxin is ingested with the results described elsewhere.

Pathogenicity for animals. While man is considerably more susceptible to infection with staphylococci than the lower animals, naturally occurring enzootic infections in domestic animals are well known, and experimental animals may be infected. In horses and cattle *Staph. aureus* is associated with pathological processes and conditions similar to those it produces in man. Staphylococcus mastitis in cattle is not uncommon; the cattle are often infected by man, or the infection is carried from one cow to another by the milker, and infected cattle constitute a minor source of human infection. Staphylococcus infection of lambs is associated with tick bite and assumes an acute and a chronic form; the first is bacteremia with death occurring within 24 hours, and the second is characterized by the development of metastatic abscesses. Staphylococci have also been isolated from naturally occurring abscesses in birds.

Of the experimental animals, the rabbit is one of the most susceptible.[36, 37, 51] Intravenous inoculation of 0.1 ml. of a 24-hour broth culture produces an infection that is fatal in four to eight days, and on autopsy abscesses are found in various organs, most commonly in the cortex of the kidney and in the walls of the heart. Usually infection does not occur in the bone marrow or periosteum, but it has been reported that osteomyelitis may be produced in young animals. If the bone is fractured or the periosteum is injured prior to inoculation, however, a sequence of events closely similar to osteomyelitis in man is produced. Ocular infections may be produced, but intraperitoneal infection is relatively unsuccessful. The mouse also is resistant to intraperitoneal inoculation, but following intravenous inoculation with approximately forty million microorganisms, about 85 per cent of the animals die, and on autopsy abscesses in the kidney are the characteristic finding. Of the other common experimental animals, the guinea pig is relatively resistant to infection with staphylococci, and the rat and pigeon are highly resistant. In general, there seems to be no satisfactory animal test for the virulence of staphylococci.

Bacteriological Diagnosis. The isolation of staphylococci is usually not a difficult matter. Blood agar is the medium of choice for isolation of the organism from purulent specimens, and on 24 hours' incubation *Staph. aureus* gives good growth of creamy, deeply pigmented colonies that are surrounded by the clear zones of β-hemolysis. The staphylococci are able to grow in the presence of 7.5 per cent sodium chloride, which is inhibitory to many bacteria, and selective mediums containing salt together with mannitol and sometimes phenol red as an acid-base indicator have been used for the isolation of staphylococci from specimens heavily

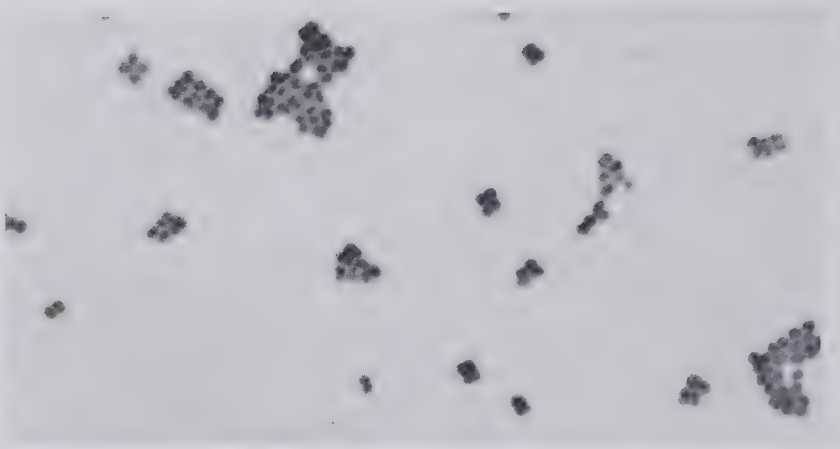


Figure 81. *Micrococcus tetragenus;* smear from pure culture stained with fuchsin. Note the relatively large size of the cells and the typical tetrad arrangement with the irregular clumps tending to be made up of tetrads. × 1800.

contaminated with other bacteria.[41, 88]

Examination of Gram-stained smears from typical colonies will show the characteristic morphology, and the coagulase test is routinely carried out, usually as the slide test in which the bacteria are suspended in plasma and observed under the microscope for clumping. Biochemical reactions, with the possible exception of mannitol fermentation and gelatinase formation, are uninformative. Differentiation of the lysins is carried out only for special purposes and most simply by inclusion of appropriate antiserums in a blood agar medium. Demonstration of enterotoxin formation requires the feeding of rhesus monkeys or human volunteers and is done only rarely; it is generally regarded as a fair presumption that staphylococci are responsible for food poisoning if hemolytic, coagulase-positive *Staph. aureus* is found in pure culture or in very large numbers in a food incriminated by epidemiological evidence.

Immunity. Acquired immunity to infection with staphylococci is generally of a low order. Both natural and acquired immunity appear to be primarily cellular in nature, with phagocytosis of the microorganisms as the most significant factor. As pointed out above, the close association of coagulase with virulence suggests that this activity is an important element in the pathogenicity of the microorganism, but it is a poor antigen, stimulating only an irregular and low-grade immune response. The staphylolysins are good antigens, however, and the immune response to α-lysin is doubtless responsible for the observations that antileucocidin is a significant part of effective acquired immunity. In addition, the nature of the infectious process is such as to minimize contact of the staphylococcal antigens with both the antibody-forming cells and the circulating antibody. Microorganisms present in the abscesses produced by these bacteria are effectively separated from the blood stream; it is well known, for example, that staphylococcal osteomyelitis even of long standing does not result in an effective immune response, and, conversely, such lesions are not affected to an appreciable extent by circulating antibody or by drugs. Even on hematogenous spread of infection with the formation of secondary abscesses, many of the viable staphylococci are protected to a certain extent from antibody that may be present in the blood, for they are contained in leucocytes or within masses of fibrin.

Active immunization procedures, making use of vaccines, often autogenous, or cell-free filtrate containing staphylolysins toxoided by treatment with formalin, are usually carried out on a therapeutic rather than prophylactic basis. Skin in-

fections, such as pustular acne, are frequently difficult to treat, and active immunization has been of interest as a therapeutic measure. In general encouraging, though not striking, results have been obtained but may not appear for some months. The therapeutic use of antiserums has given disappointing results.

Chemotherapy. Sensitive strains of staphylococci are susceptible to the antibacterial activity of the sulfonamides, penicillin, the tetracyclines, chloramphenicol, erythromycin, and other antibiotics active on gram-positive bacteria. The 80 per cent case fatality rate in staphylococcal septicemia was reduced by about 30 per cent with the introduction of penicillin, but subsequent development of drug-resistant strains has increased this over-all figure to 50 per cent or more. The problem of chemotherapy of staphylococcal infections is, therefore, complicated by the prevalence of resistant strains, and strains of multiple resistance, and resistance to new antibiotics develops rapidly as they come into use. It is essential, therefore, that strains isolated from pathological processes be tested for sensitivity to a variety of potentially effective agents as a part of routine diagnostic procedure so that a basis for rational and effective chemotherapy may be provided.

When the strain is sensitive to an available chemotherapeutic agent, chemotherapy of staphylococcus bacteremia is strikingly successful, of localized infection in the form of abscesses less so, and of osteomyelitis not at all without adequate surgery. In the last two, of course, the microorganisms are partially or practically completely protected from the drug.

OTHER MICROCOCCI

A variety of micrococci, both pigmented and otherwise, have been described, most of which are saprophytic forms found in water and elsewhere in nature. A well-known representative of this group is *Sarcina lutea*, a coccal form producing a bright yellow pigment which derives its generic name from a tendency to form cubical packets of eight cells.

Micrococcus tetragenus (*Gaffkya tetragena*) is a parasitic coccus frequently found on the mucous membranes of the upper respiratory tract. It was discovered by Gaffky in 1881 in the pulmonary cavities in phthisis and has been found in pure culture in abscesses in animals and man, and often occurs in the healthy mouth. It is not uncommonly found in suppurations of the mouth and neck. It is also found in the empyema following pneumonia and in the pus of war wounds. This microorganism is probably of low-grade virulence and unable, as a rule, to invade the human

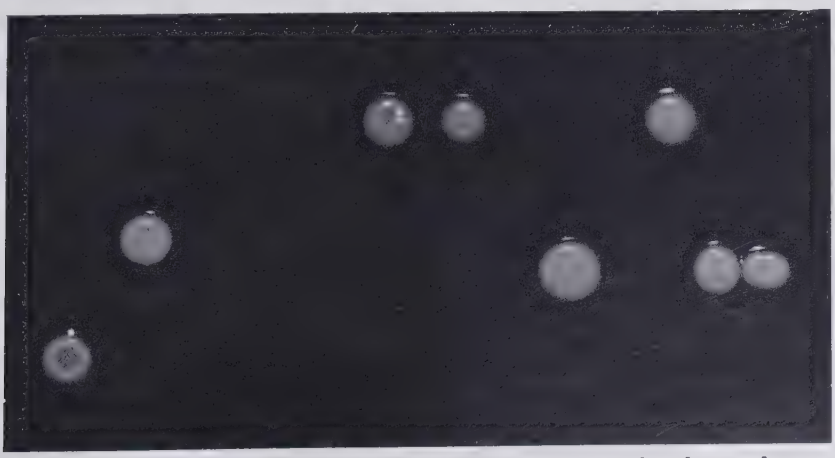


Figure 82. Colonies of *Micrococcus tetragenus* on blood agar. Twenty-four-hour culture; × 3.

tissues except when the resistance is lowered by some depressing influence, especially of the kind caused by the invasion of some other bacterium. White mice inoculated with *M. tetragenus* succumb to a rapidly progressing septicemia. Guinea pigs and rabbits usually show only a local affection. House mice and rats are relatively resistant.

Morphologically *M. tetragenus* is distinguished by its occurrence in tetrads or groups consisting of four small oval cocci. It is gram-positive. In cultures the sheet-like arrangement is not always seen, but in the animal organism the flat tablets occur uniformly, and a rather heavy capsule surrounds the tetrad. On agar a confluent rough, elevated white growth is produced. Gelatin is not liquefied; milk is coagulated. Growth is slow and occurs at 20° and at 37° C., though better at the higher temperature.

REFERENCES

1. Allison, V. D.: Discussion on food poisoning. Proc. Roy. Soc. Med., 1949, **42:**214–220.
2. Anderson, K.: The effect of staphylococcal filtrates on isolated rabbit small intestine, with special reference to enterotoxic strains of *Staph. pyogenes.* Brit. J. Exptl. Path., 1953, **34:**548–555.
3. Barber, M., et al.: Reversal of antibiotic resistance in hospital staphylococcal infections. Brit. Med. J., 1960, **i:**11–17.
4. Blair, J. E.: The pathogenic staphylococci. Bact. Rev., 1939, **3:**97–146.
5. Blair, J. E.: Epidemiological implications of staphylococcal phage typing. Ann. N. Y. Acad. Sci., 1956, **65:**152–159.
6. Blair, J. E.: Factors determining the pathogenicity of staphylococci. Ann. Rev. Microbiol., 1958, **12:**491–506.
7. Blair, J. E., and Carr, M.: The bacteriophage typing of staphylococci. J. Infect. Dis., 1953, **93:**1–13.
8. Blair, J. E., and Carr, M.: Distribution of phage groups of *Staphylococcus aureus* in the years 1927 through 1947. Science, 1960, **132:**1247–1248.
9. Blair, J. E., and Williams, R. E. O.: Phage typing of staphylococci. Bull. W. H. O., 1961, **24:**771–784.
10. Blobel, H., and Berman, D. T.: Further studies on the in vivo activity of staphylocoagulase. J. Infect. Dis., 1961, **108:**63–67.
11. Blobel, H., Berman, D. T., and Simon, J.: Purification of staphylococcal coagulase. J. Bact., 1960, **79:**807–815.
12. Boniece, W. S., Wick, W. E., and Holmes, D. H.: White variants derived from *Staphylococcus aureus:* Comparison with parent strains. J. Bact., 1957, **73:**685–686.
13. Burns, J., and Holtman, D. F.: Biochemical properties of virulent and avirulent staphylococci. Ann. N. Y. Acad. Sci., 1960, **88:**1115–1124.
14. Casman, E. P.: Further serological studies of staphylococcal enterotoxin. J. Bact., 1960, **79:**849–856.
15. Cohn, Z. A., and Morse, S. I.: Interactions between rabbit polymorphonuclear leucocytes and staphylococci. J. Exptl. Med., 1959, **110:**419–443.
16. Dack, G. M.: Food Poisoning. University of Chicago Press. 3rd ed., 1956.
17. Dearing, W. H., and Needham, G. M.: Hospitalized patients with *Staphylococcus aureus* in the intestine. J. Amer. Med. Assn., 1960, **174:**1597–1602.
18. Dunnet, W. N., and Schallibaum, E. M.: Scarlet fever-like illness due to staphylococcus infection. Lancet, 1960, **ii:**1227–1229.
19. Duthie, E. S.: Variation in the antigenic composition of staphylococcal coagulase. J. Gen. Microbiol., 1952, **7:**320–326.
20. Duthie, E. S.: Evidence for two forms of staphylococcal coagulase. J. Gen. Microbiol., 1954, **10:**427–436.
21. Duthie, E. S.: The production of free staphylococcal coagulase. J. Gen. Microbiol., 1954, **10:**437–444.
22. Eksted, R. D.: Further studies on the antibacterial activity of human serum on *Micrococcus pyogenes* and its inhibition by coagulase. J. Bact., 1956, **72:**157–161.
23. Eksted, R. D., and Nungester, W. J.: Coagulase in reversing antibacterial activity of normal human serum on *Micrococcus pyogenes.* Proc. Soc. Exptl. Biol. Med., 1955, **89:**90–94.
24. Elek, S. D.: Staphylococcus Pyogenes and its Relation to Disease. Williams and Wilkins, Baltimore. 1959.
25. Elek, S. D., and Levy, E.: Distribution of hemolysins in pathogenic and non-pathogenic staphylococci. J. Path. Bact., 1950, **62:**541–554.
26. English, A. R., McBride, T. J., and Huang, H. T.: Microbial resistance to penicillin as related to penicillinase or penicillin acylase activity. Proc. Soc. Exptl. Biol. Med., 1960, **104:**547–549.
27. Evans, J. B., and Niven, C. F., Jr.: A comparative study of known food-poisoning staphylococci and related varieties. J. Bact., 1950, **59:**545–550.
28. Fahlberg, W. J., and Marston, J.: Coagulase production by *Staphylococcus aureus.* Factors affecting coagulase production. J. Infect. Dis., 1960, **106:**111–115.
29. Fairbrother, R. W., and Taylor, G.: Sodium Methicillin in routine therapy. Lancet, 1961, **i:**473–476.
30. Finland, M.: Emergence of antibiotic-resistant bacteria. New England J. Med., 1955, **253:**909–922; 969–979; 1019–1028.
31. Finland, M., Jones, W. F., and Bennett, I. L., Jr.: Antibiotic susceptibility and phage types of pathogenic staphylococci. A study of two hundred ten strains isolated at Boston City Hospital in 1955. Arch. Intern. Med., 1959, **104:**365–377.
32. Fisher, A. M., et al.: Staphylococcal pneumonia. A review of 21 cases in adults. New England J. Med., 1958, **258:**919–928.

33. Gillespie, W. A., Simpson, K., and Tozer, R. C.: Staphylococcal infection in a maternity hospital. Epidemiology and control. Lancet, 1958, **ii:**1075–1080.
34. Gladstone, G. P.: The nutrition of *Staphylococcus aureus:* Nitrogen requirements. Brit. J. Exptl. Path., 1937, **18:**322–333.
35. Gladstone, G. P., and van Heyingen, W. E.: Staphylococcal leucocidins. Brit. J. Exptl. Path., 1957, **38:**123–137.
36. Goshi, K., Cluff, L. E., Johnson, J. E., and Conti, C. R.: Studies on the pathogenesis of staphylococcal infection. II. The effect of nonspecific inflammation. J. Exptl. Med., 1961, **113:**249–257.
37. Goshi, K., Cluff, L. E., and Johnson, J. E.: Studies on the pathogenesis of staphylococcal infection. III. The effect of tissue necrosis and antitoxic immunity. J. Exptl. Med., 1961, **113:** 259–270.
38. Gresham, G. A.: Staphylococcal pneumonia. Brit. J. Clin. Practice, 1958, **12:**247–252.
39. Grün, L.: Die serologische Differenzierung von Staphylokokken. Ztschr. f. Hyg. u. Infektionskr., 1959, **146:**129–141.
40. Hinton, N. A., Taggart, J. G., and Orr, J. H.: The significance of the isolation of coagulase-positive staphylococci from stool. With special reference to the diagnosis of staphylococcal enteritis. Amer. J. Clin. Path., 1960, **33:** 505–510.
41. Hinton, N. A., Taggart, J. G., and Orr, J. H.: Diagnosis of staphylococcal enteritis. Canadian Med. Assn. J., 1960, **83:**700–704.
42. Hunt, G. A., and Moses, A. J.: Acute infection of mice with Smith strain of *Staphylococcus aureus. Science,* 1958, **128:**1574–1575.
43. Innes, A. G.: Tellurite-egg agar, a selective and differential medium for the isolation of coagulase-positive staphylococci. J. Applied Bact., 1960, **23:**108–113.
44. Jacherts, D.: Experimentelle Untersuchungen über die Identität freier und gebundener Coagulase. Ztschr. f. Hyg. u. Infektionskr., 1956, **142:**502–509.
45. Jackson, A. W., and Little, R. M.: Staphylococcal toxins. I. Factors affecting the hemolytic activity of α-toxin. Canadian J. Microbiol., 1957, **3:**47–54.
46. Jackson, A. W., and Little, R. M.: Leucocidal effect of staphylococcal δ-toxin. Canadian J. Microbiol., 1957, **3:**101–102.
47. Jackson, A. W., and Little, R. M.: Staphylococcal toxins. II. Factors affecting hemolysis by δ-lysin. Canadian J. Microbiol., 1958, **4:**435–444.
48. Jackson, A. W., and Little, R. M.: Staphylococcal toxins. III. Partial purification and some properties of δ-lysin. Canadian J. Microbiol., 1958, **4:**453–461.
49. Jackson, A. W., and Mayman, D.: Staphylococcal toxins. IV. Factors affecting hemolysis by β-lysin. Canadian J. Microbiol., 1958, **4:**477–486.
50. Jackson, J. R., Gibbons, R. J., and Magner, D.: The effects of staphylococcal toxin on the lungs of rabbits. Amer. J. Path., 1958, **34:** 1051–1067.
51. Johnson, J. E., Cluff, L. E., and Goshi, K.: Studies on the pathogenesis of staphylococcal infection. I. The effects of repeated skin infections. J. Exptl. Med., 1961, **113:**235–248.
52. Kimler, A.: Some clinical laboratory briefs on staphylococci. J. Bact., 1962, **83:**207–208.
53. Knox, R.: A new penicillin (BRL 1241) active against penicillin-resistant staphylococci. Brit. Med. J., 1960, **ii:**690–693.
54. Lack, C. H.: Staphylokinase: An activator of plasma protease. Nature, 1948, **161:**559–560.
55. Lack, C. H.: Biological characteristics of staphylococci recovered from pathologic materials. Ann. N. Y. Acad. Sci., 1956, **65:**103–108.
56. Lack, C. H., and Wailling, D. G.: A study of 435 strains of *Staphylococcus pyogenes* with reference to factors which may contribute to pathogenicity. J. Path. Bact., 1954, **68:**431–443.
57. Maitland, H. B., and Martyn, G.: A selective medium for isolating staphylococcus based on the differential inhibiting effect of increased concentrations of sodium chloride. J. Path. Bact., 1948, **60:**553–561.
58. Marston, J., and Fahlberg, W. J.: Coagulase production by *Staphylococcus aureus.* II. Growth and coagulase production in complex and chemically defined mediums—comparison of chemically defined mediums. J. Infect. Dis., 1960, **106:**116–122.
59. Martin, W. J., Nichols, D. R., and Henderson, E. D.: The problem of management of nasal carriers of staphylococci. Proc. Staff Meetings Mayo Clinic, 1960, **35:**282–292.
60. McDermott, W.: The problem of staphylococcal infection. Brit. Med. J., 1956, **ii:**837–840.
61. Melly, M. A., Thomison, J. B., and Rogers, D. E.: Fate of staphylococci within human leukocytes. J. Exptl. Med., 1960, **112:**1121–1130.
62. Munch-Peterson, E.: Staphylococcal carriage in man: An attempt at a quantitative survey. Bull. W. H. O., 1961, **24:**761–770.
63. Nahmias, A., Sakurai, N., Blumberg, R., Doege, A., and Sulzer, C.: The staphylococcus "80/81 complex": Epidemiological and laboratory observations. J. Infect. Dis. 1961, **109:**211–222.
64. Negro, R. C., Gentile-Ramos, I., and Galiana, J.: Escarlatina por *Staphylococcus aureus.* An. Fac. de Med. Montevideo, 1956, **41:**263–273.
65. Oeding, P.: Antigenic properties of *Staphylococcus aureus.* Bact. Rev., 1960, **24:**374–396.
66. Oeding, P., and Williams, R. E. O.: The type classification of *Staphylococcus aureus:* A comparison of phage-typing with serological typing. J. Hyg., 1958, **56:**445–454.
67. Palmer, E. D.: The morphologic consequences of acute exogenous (staphylococcic) gastroenteritis on the gastric mucosa. Gastroenterology, 1951, **19:**462–475.
68. Panton, P. N., and Valentine, F. C. O.: Staphylococcal toxin. Lancet, 1932, **i:**506–508.
69. Pillet, J., Calmels, J., Orta, B., and Chabanier, G.: Étude comparée du type sérologique de la sensibilité aux bactériophages et l'antibiogramme de 201 souches de staphylococcus isolés par prélèvements systématiques chez la nourrisson. Ann. Inst. Pasteur, 1954, **86:**309–319.
70. Rammelkamp, C. H., Jr., and Lebovitz, J. L.: The role of coagulase in staphylococcal infections. Ann. N. Y. Acad. Sci., 1956, **65:**144–151.
71. Robertson, L., Caley, J. P., and Moore, J.: Importance of *Staphylococcus aureus* in the 1957 epidemic of influenza A. Lancet, 1958, **ii:**233–236.
72. Robinson, J., Thatcher, F. S., and Montford, J.: Studies with staphylococcal toxins. V. Possible identification of alpha hemolysin with a proteolytic enzyme. Canadian J. Microbiol., 1960, **6:**183–194.

73. Robinson, J., Thatcher, F. S., and Montford, J.: Studies with staphylococcal toxins. VI. Some properties of a nonhemolytic dermonecrotic toxin produced by some strains of *Staphylococcus aureus*. Canadian J. Microbiol., 1960, **6:**195–201.
74. Rogers, D. E.: The blood stream clearance of staphylococci in rabbits. Ann. N. Y. Acad. Sci., 1956, **65:**73–84.
75. Rogers, D. E.: Current problems in staphylococcal infections. Ann. Intern. Med., 1956, **45:** 748–781.
76. Rutenburg, A. M., Greenburg, H. L., and Schweinburg, F. B.: Clinical experiences with 2,6-dimethoxyphenyl penicillin monohydrate in staphylococcal infections. New England J. Med., 1960, **263:**1174–1178.
77. Schwabacher, H., Cunliffe, A. C., Williams, R. E. O., and Harper, G. J.: Hyaluronidase production by staphylococci. Brit. J. Exptl. Path., 1945, **26:**124–129.
78. Shooter, R. A., et al.: Spread of staphylococci in a surgical ward. Brit. Med. J., 1958, **i:**607–613.
79. Simpson, J.: Staphylococcal scarlet fever. Med. Officer, 1953, **89:**85–87.
80. Smith, D. D., and Johnstone, J. M.: Coagulase activity in vivo. Brit. J. Exptl. Path., 1958, **39:** 165–170.
81. Smith, I. M., and Vickers, A. B.: Natural history of 338 treated and untreated patients with staphylococcal septicemia (1936–1955). Lancet, 1960, **i:**1318–1322.
82. Sugiyama, H., Bergdoll, M. S., and Dack, G. M.: In vitro studies on staphylococcal enterotoxin production. J. Bact., 1960, **80:**265–270.
83. Surgalla, M. J., Bergdoll, M. S., and Dack, G. M.: Use of antigen-antibody reactions in agar to follow the progress of fractionation of antigenic mixtures: Application to purification of staphylococcal enterotoxin. J. Immunol., 1952, **69:**357–365.
84. Surgalla, M. J., Bergdoll, M. S., and Dack, G. M.: Staphylococcal enterotoxin: Neutralization by antiserum. J. Immunol., 1954, **72:**398–403.
85. Symposium: Symposium on carriers of penicillin-resistant staphylococci outside hospital. Proc. Roy. Soc. Med., 1960, **53:**253–260.
86. Tager, M.: Problems in the coagulation of plasma by staphylocoagulase. Ann. N. Y. Acad. Sci., 1956, **65:**109–118.
87. Thompson, R. E. M., Whitby, J. L., and Harding, J. W.: Treatment of experimental penicillin-resistant staphylococcal lesions with BRL 1241. Brit. Med. J., 1960, **ii:**706–708.
88. Vogel, R. A., and Johnson, M.: Modification of the tellurite-glycine medium for use in the identification of *Staphylococcus aureus*. Pub. Health Lab., 1960, **18:**131–133.
89. Wallmark, G., and Finland, M.: Phage types and antibiotic susceptibility of pathogenic staphylococci. Results at Boston City Hospital 1959–1960 and comparison with strains of previous years. J. Amer. Med. Assn., 1961, **175:**886–897.
90. Webster, C. U.: Staphylococcal diarrhoea. Lancet, 1958, **ii:**1036–1038.
91. Wilson, J. B.: Comparative susceptibility of chimpanzees and *Macaca mulatta* monkeys to oral administration of partially purified staphylococcal enterotoxin. J. Bact., 1959, **78:**240–242.
92. Wissler, H.: Die Staphylokokkenpneumonie des Kindes. Schweiz. med. Woch., 1961, **91:**385–389; 416–419.
93. Yotis, W. W., and Eksted, R. D.: Studies on staphylococcus. I. Effect of serum and coagulase on the metabolism of coagulase positive and coagulase negative strains. J. Bact., 1959, **78:**567–574.
94. Zebovitz, E., Evans, J. B., and Niven, C. F., Jr.: Tellurite-glycine agar: a selective plating medium for the quantitative detection of coagulase-positive staphylococci. J. Bact., 1955, **70:** 686–690.

Chapter 17

THE STREPTOCOCCI

THE STREPTOCOCCI make up a relatively large group of pyogenic coccus forms characterized by an arrangement of the cells in chains. They are responsible for a variety of diseases of man and certain diseases of lower animals, and some are saprophytes found in milk and milk products. They were early observed in the pus formed in suppurative inflammatory conditions, and their frequent presence and pathological significance were first emphasized by Ogston, by Fehleisen, and by Rosenbach in the early 1880's. It is now well known that, in addition to the more virulent pathogenic forms, relatively harmless parasitic streptococci are more or less constantly present in the human throat and in the intestinal tract which assume a pathogenic role only under circumstances in which normal resistance is markedly reduced, and which may be regarded, for all practical purposes, as a portion of the normal flora of the human body.

Morphology and Staining. Like staphylococci, individual streptococci are spherical and 0.8 to 1.0 μ in diameter. Some variation in size results from the character of the culture medium, and the individual cells are frequently appreciably smaller when the cultures are grown under anaerobic conditions. Smaller varieties, 0.4 to 0.8 μ in diameter, whose size is apparently a constant character, have been described. The typical streptococcus divides in only one plane, and the tendency of the cells to remain united results in the development of the characteristic chains that give these organisms their generic name. This tendency is apparently more pronounced between daughter cells following the first cell division, and the chains frequently have the appearance of chains of diplococci, with pairs closer to one another than to adjacent pairs; this is shown in Figure 83. The firmness of the attachment is to some degree a strain characteristic, and some strains appear as relatively long chains while others show little more than two pairs of diplococci.

Earlier workers attached some importance to the length of chains formed and differentiated a supposedly more virulent *Streptococcus longus* and a less virulent *Str. brevis*. This distinction has little meaning, in spite of the fact that freshly isolated streptococci from pathological processes usually form chains of more than eight cells while those normally present in the mouth and throat usually develop only short chains. For instance, longer chains are generally formed during growth in liquid mediums; *Str. lactis*, a common inhabitant of milk, forms very long chains, and short-chain streptococci are not infrequently isolated from pathological conditions. The formation of chains of cells is in no sense absolute, however, and on microscopic examination of a smear of typical streptococci, single cells, pairs of cells, and occasional aggregates resembling staphylococci are found.

Streptococci are not motile under ordi-

nary conditions of observation, but motile forms have been described from time to time.[39] The majority of strains are encapsulated, and in some the capsular material consists of hyaluronic acid, the substrate of hyaluronidase. So far as is known, these bacteria do not form spores, and the formation of pigment is relatively rare.

They stain readily with the usual bacterial stains. Almost all of the strains isolated from pathological processes in man are gram-positive, but gram-negative streptococci are found, more commonly in suppurative conditions in lower animals than in man.

Streptococcus colonies on agar mediums are usually quite small, translucent, convex, entire, and slightly granular. The colonial form designated matt, with a ground-glass rather than a glistening or glossy surface, is the virulent form of β-hemolytic streptococci commonly encountered on primary isolation from infectious processes, but variant forms occur also (see below).

Physiology. As a group, the streptococci grow over a relatively wide temperature range, 10° to 42° C. Those of the pyogenic group, which is made up of human and animal parasites, have an optimum at 37° C. and are relatively restricted in range, those of the lactic group grow over 10° to 37° C., and the viridans group grows from 37° to 42° C. and includes one thermophilic species which grows at 50° C. The majority of streptococci are facultative anaerobes, but there are a few obligate anaerobic varieties.

The streptococci are among the more fastidious of bacteria with respect to nutritive requirements. They will usually not grow on meat extract mediums, and growth is ordinarily poor even on infusion mediums but may be somewhat improved by the inclusion of phosphate buffer and a small amount, perhaps 0.1 per cent, of glucose. For the most part infusion mediums enriched by the addition of 10 per cent defibrinated blood, ascitic fluid, and similar substances are used, and a medium such as blood agar is satisfactory for routine culture of the pathogenic forms. Many strains are hemolytic on blood agar, some showing the clear zones of β-hemolysis, and others a zone of greenish discoloration or α-hemolysis indistinguishable from that produced by the pneumococcus. Growth also occurs in milk which is curdled by some species because of the fermentation of lactose.

Streptococcus cultures may be preserved in serum broth or agar or infusion gelatin stabs in the refrigerator.

The relatively complex nutritive requirements of these bacteria have been defined to a considerable degree. Most strains require glutamine, riboflavin, pantothenic acid, pyridoxine, nicotinic acid, and biotin, together with 13 or 14

Figure 83. *Streptococcus pyogenes.* Recently isolated scarlet fever strain. Note the tendency to diplococcus arrangement in the chains. Fuchsin; × 1050.

amino acids. The streptococci of group A also require nucleic acid derivatives.[127] Some strains have been cultivated in mediums containing albumin or casein hydrolysate, supplemented with various amino acids and vitamins, and others in chemically defined mediums.[28, 69]

A wide variety of sugars are fermented and a number of polysaccharides are hydrolyzed. The chief fermentation product of glucose is lactic acid, and small amounts of formic and acetic acids and ethyl alcohol are formed. The hydrolysis of sodium hippurate and polymers such as inulin, starch, and dextrin has some differential significance, together with the fermentation of lactose, sorbitol, glycerol, mannitol, maltose, sucrose, and raffinose. Some strains liberate relatively large amounts of ammonia from peptone, and this characteristic also has some differential value. With rare exceptions, inulin is not fermented nor are the streptococci dissolved in ox bile or a 10 per cent solution of bile salt; these characteristics have considerable practical importance in that they serve to differentiate the α-hemolytic or green streptococci from the pneumococci.

The Formation of Toxic Substances. The hemolytic streptococci produce a number of toxic substances. Certain of these appear to be intracellular and are found in sonic lysates of the bacteria, while others diffuse from the cells and are found in cell-free filtrates. In the former group, an intracellular hemolysin is formed which is demonstrable in sonic lysates,[91, 92, 95] is distinct from the extracellular streptolysins (see below), and is lethal for the mouse.[100] Intracellular substances which are pyrogenic, produce rash, and give nodular lesions in the dermal connective tissue of rabbits have been studied at length.[13, 15, 93, 118] These activities appear to be associated with the cell wall[87] and contain active polysaccharide,[94] but are differentiated from the endotoxins of the gram-negative bacilli.[14]

The extracellular toxic activities include the streptolysins or hemolysins, streptokinase, hyaluronidase, the erythrogenic or scarlatinal toxin, leucocidin which kills heterophils which have ingested the bacteria,[128] and possibly an enterotoxin in the case of certain of the α-hemolytic streptococci which are an occasional cause of food-poisoning.[70]

Streptolysins. Two kinds of filterable hemolysin produced by streptococci were distinguished by Todd. They differ in that one, called streptolysin S, is sensitive to treatment with heat or acid, and the other, which is designated streptolysin O, is inactivated by oxygen, *i.e.*, is inactive in the oxidized state, and the activity may be restored by treatment with mild reducing agents such as sulfite. Both hemolysins are extremely labile at 37° C. and disappear rapidly after the first few hours of incubation.

Streptolysin O appears to be much the more important to the virulence of the hemolytic streptococci, and has a specific cardiotoxic activity which has been described by Bernheimer and his associates.[7, 8, 46] Inoculation of the toxin results in the release of inhibitor, lipoprotein in nature, from the heart muscle of the frog resulting in sensitivity to the cardiotoxic action, and which is also released to occur in the serum albumin fraction and inhibits hemolysis *in vitro*.[90] There is also evidence that streptolysin O has leucocidin activity. This toxin is antigenic, and antibody to it is regularly formed in streptococcal infections; the antibody titration has been put upon a standardized basis.[106]

Streptokinase.[113] The hemolytic streptococci of groups A, C, and G (see below) produce streptokinase, the plasmin activator that initiates the fibrinolytic dissolution of fibrin clots (Chap. 9). It is most actively produced by strains of group C and only irregularly and to low titer by strains of group G. No special cultural conditions seem to be required for the production of the activity. There is some species specificity in that human, dog, and rabbit plasminogens are readily activated, but those of pig, horse, and cow are less readily activated, and guinea pig and sheep plasminogens are not activated by the usual concentrations of streptokinase.[1] The activity is antigenic and antibody is produced, seem-

ingly irregularly, in infected persons.[79] The titration of the antibody is complicated by variation in the antiprotease and lytic factor concentrations in the test plasma, and, more important, streptokinase is not immunologically homogeneous and that produced by different strains of streptococci is often antigenically different.[120] Titration of such antibody levels has little significance in practice.

Hemolytic streptococci also produce streptodornase, a deoxyribonuclease which is apparently unrelated to virulence. It is not homogeneous, even within a single streptococcal strain, and has been separated electrophoretically into three components, A, B, and C; the last is inhibited by citrate and all three by chelating agents.[117] Streptokinase and streptodornase are available as commercial preparations (Varidase) for clinical application in the enzymatic debridement of necrotic tissue and dissolution of fibrous exudates.

Hyaluronidase. The majority of hemolytic streptococci produce hyaluronidase, the enzymatic activity of which depolymerizes the ground substance of the tissues (Chap. 9). Strains isolated from healthy throats produce as much hyaluronidase as those from mild or severe infections.[16] The capsular substance of encapsulated strains of hemolytic streptococci of both groups A and C consists of hyaluronic acid and such strains do not produce hyaluronidase although they are usually virulent.[61] It is a plausible supposition that the production of this activity may be causally related to the ability of virulent streptococci to spread within the tissues, and in fact the production of hyaluronidase is increased in the presence of the substrate. The virulence of encapsulated strains and the lack of direct experimental evidence would seem to invalidate such an assumption. On the contrary, the production of hyaluronic acid, *e.g.*, capsules, while apparently unimportant to experimental infection by the intraperitoneal route, appears to be essential to infection by inhalation.[17, 42]

Erythrogenic Toxin. The erythrogenic toxin is a substance which gives rise to a marked local erythema upon intradermal inoculation in man, and in larger amounts produces a generalized erythematous rash. A skin reaction may be produced in the rabbit, but, in general, laboratory animals are highly or completely resistant to it. The lethal dose for the rabbit is very large, 5 to 10 ml. of unconcentrated filtrate. This toxin is responsible for the rash of scarlet fever and is known as "scarlatinal toxin," or "Dick toxin" after its discoverers. It differs from the classic exotoxins in that it is relatively heat-resistant and some toxicity still remains after boiling for 30 minutes. It is antigenic and stimulates the production of specific antitoxin but not to the high titers readily obtained for diphtheria and tetanus antitoxin.

The formation of the foregoing substances by the streptococci is a characteristic of the group rather than of all strains of pathogenic streptococci. Thus all group A streptococci do not produce erythrogenic toxin, and those that do not are incapable of causing scarlet fever. In general they are associated with virulence in that avirulent varieties are frequently nonhemolytic, nonfibrinolytic, and the like, while those found in pathological conditions and showing high virulence under experimental conditions are hemolytic, fibrinolytic, etc.

Hypersensitivity. Another factor associated with the ability of streptococci to produce disease is the development of a hypersensitivity to the cell substance of these bacteria during and following infection. Subsequent infections, then, result in allergic phenomena which may be of considerable importance in the disease produced. It seems probable that hypersensitivity plays a part in rheumatoid disease and arthritis of streptococcal etiology (see below).

Variation. Alterations in the morphology of individual streptococci are frequently observed in old cultures, with cells swollen to several times normal size. These and other changes in aging cultures have been interpreted by some workers as indicative of a complex life cycle, but it is more likely that such

aberrant morphology is that of involution forms, *i.e.*, is degenerative in nature. The streptococci may, under appropriate conditions, occur as L forms and protoplasts.[27] These occur in group C as well as group A hemolytic streptococci.[68]

Dissociative changes in colonial morphology are well known. A form designated as matt is the virulent form and distinct from the usual smooth and rough colony types. A mucoid colony type has been described which was still different. There are, then, four recognized colonial types: smooth (glossy), rough, mucoid, and matt. The conversion from mucoid or matt to rough or smooth corresponds to the usual S → R dissociative change. The M antigen is present in the matt colony type, in fact derives its name from the association, but is deficient in the smooth glossy colonial type. Exceptions are not uncommon, and colonial morphology appears to be determined primarily by capsule formation, *i.e.*, mucoid or matt colonies are formed by encapsulated streptococci and the smooth, glossy colony by strains which do not form them; the occurence of M antigen is associated with capsule formation rather than contributing to colonial type.[129] Other immunological variation, not correlated with colonial form, may occur also, for there is some evidence that the agglutinative types are sometimes unstable and that on occasion the group-specific polysaccharide may be lost (see below).

Variation in hemolysis is commonly reported in which β-hemolytic strains give rise to nonhemolytic or α-hemolytic variants. The alteration in hemolysis is to some degree an environmental effect in that anhemolytic variants may be hemolytic under anaerobic conditions, suggesting an inactivation of oxygen-labile hemolysin rather than failure to produce it; similarly α-hemolytic variants may be made β-hemolytic by including catalase in the medium or omitting reducing sugar, since the latter appears to inhibit hemolysin production by some strains of streptococci. Streptolysin O is generally regarded as responsible for blood plate hemolysis, but anhemolytic variants of β-hemolytic strains have been observed which continue to form streptolysin O in liquid culture.

Drug resistance. Streptococcal infections are successfully treated with sulfonamides, penicillin, and the broad-spectrum antibiotics but may become resistant to these agents. Resistance may be induced experimentally in the usual way, and drug-resistant strains have been found to occur under natural conditions. As a group, the streptococci are much less prone to become resistant under natural conditions than are the staphylococci, and strains of acquired drug resistance do not occur often enough to present any practical problem.[35] Although the β-hemolytic streptococci are relatively uniform in their susceptibility to the chemotherapeutic drugs, the α-hemolytic forms vary widely, but as a strain rather than an induced characteristic.

That resistant strains may occur on a relatively large scale was demonstrated during World War II when sulfadiazine was used for the prophylaxis of streptococcal infection in certain military training camps.[36] The resistant strain, or strains, that emerged spread in the military population and into the civilian population, but with discontinuance of the general use of sulfonamides when penicillin, and subsequently other antibiotics, became generally available, sulfadiazine resistance became of no importance and seems to have disappeared. On the other hand, the widespread use of penicillin, both generally and for the prophylaxis of rheumatic fever (see below), has not been accompanied by the general appearance of penicillin-resistant streptococci.

Classification.[101, 102] The differentiation and identification of the streptococci are a matter of very considerable practical importance because of their etiological relation to a number of widespread diseases of man and domestic animals and, of equal importance, from the theoretical point of view. It has been and continues to be a particularly difficult matter since neither the physiological nor immunological methods have

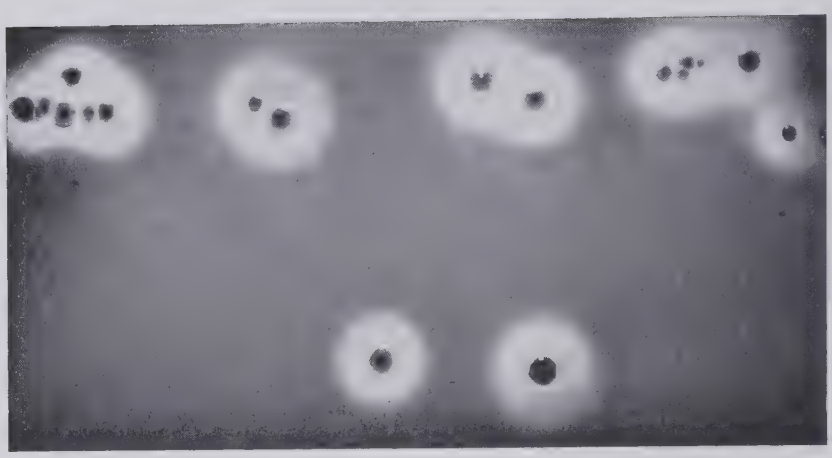


Figure 84. *Streptococcus pyogenes.* Pure culture on blood agar showing β-hemolysis. × 5.

been satisfactory. As a consequence there is basic disagreement among workers in this field as to what constitutes a species or variety and on what basis or bases differentiation should be made. Three general criteria have been used, *viz.,* hemolysis on blood agar plate culture, biochemical properties, and immunological character as indicated by precipitin and agglutination reactions. Those concerned with the pathogenic streptococci make a tentative preliminary separation on the basis of hemolysis and define species and types on an immunological basis. Workers with more general interests tend to rely primarily on physiological characters, and this is the basis of the Bergey classification.

Hemolysis. The use of blood plate hemolysis was introduced by Schottmüller in 1903 and is especially convenient since blood agar is the medium of choice in primary isolation. On this basis three types of streptococci may be distinguished:

(1) the β-hemolytic streptococci, which produce a clear zone of hemolysis in the red, opaque medium immediately surrounding the colony
(2) the α-hemolytic or green streptococci, which produce a zone of greenish discoloration in the medium about the colony which is considerably smaller than the clear zone of β-hemolysis
(3) the anhemolytic, indifferent, or γ streptococci, which produce no change in the medium

These distinctions have some validity in that the highly virulent streptococci isolated from pathological conditions are almost invariably the β-hemolytic variety. In the older literature these are grouped as a single species with the name *Str. hemolyticus,* and some workers further differentiated on the basis of the disease with which the strain was associated, *viz., Str. scarlatinae* (scarlet fever) *Str. epidemicus* (epidemic septic sore throat), *Str. erysipelatis* (erysipelas), etc. It is now quite clear that these distinctions are invalid in that identical streptococci may cause more than one clinical disease and that the same disease may be caused by immunologically distinct streptococci. At the same time, antigenic types of streptococci may be associated with one or another manifestation of streptococcal infection, such as the association of type 12 with acute glomerulonephritis; whether such associations as have been observed are more than fortuitous is not clear.

The β-hemolytic streptococci associated with diseases of lower animals do, however, show a high degree of specificity in the case of *Str. equi,* causing strangles in horses, and *Str. agalactiae,* causing mastitis in cattle, but not in that of the organism now known as *Str. zooepidemicus* which causes a wide variety of suppurative diseases in animals, including mastitis in cattle.

The β-hemolytic streptococci of human disease are usually not found in

lower animals, but may occasionally infect the udder of the cow, giving rise to milk-borne streptococcal infection. Conversely, those of animal origin do not ordinarily infect man, though human infections with *Str. zooepidemicus,* while not common, are somewhat more so than is generally believed. An even greater heterogeneity in the group is indicated by the fact that some nonpathogenic forms are also β-hemolytic. It is clear that the inclusion of all these forms under the single species *Str. hemolyticus* is not justified.

A somewhat similar situation holds true with the α-hemolytic or green streptococci which have been grouped as a single species, *Str. viridans.* The green-producing group embraces such forms as the fecal streptococci or enterococcus group, those which normally inhabit the mouth and throat, and nonpathogenic forms. Some of the α-hemolytic forms, especially those found in the throat and intestine, are able to set up disease processes if normal resistance is reduced, with the production of localized infections at the roots of teeth, in the heart valves in bacterial endocarditis, and other locations. These pathogenic forms differ rather sharply from the highly virulent β-hemolytic streptococci, but again the entire group is too heterogeneous to justify inclusion in a single species.

The anhemolytic or indifferent streptococci are almost all saprophytic forms found in milk and various dairy products. The only pathological condition with which they have been unequivocally associated is subacute bacterial endocarditis,[88] in which they have been found in a small minority of cases. Here a variety of physiologically different types are included in a single group, and, as in the case of the other groups separated on the basis of hemolysis, it is too heterogeneous to allow lumping into the single species, *Str. anhemolyticus.*

Immunological differentiation. Largely through the work of Lancefield and her co-workers, the pattern of antigenic structure of the β-hemolytic streptococci has been defined, but the viridans and anhemolytic forms are serologically diverse so that this approach has not been useful. By the extraction of soluble antigens and application of the precipitin test, Lancefield has demonstrated the presence of both group-specific and type-specific antigens in the hemolytic streptococci. The group-specific antigen, or C substance, is polysaccharide in nature and an integral part of the bacterial cell rather than a capsular material. On the basis of the specificity of this antigen five groups were originally described and designated Group A, Group B, and so on; additional groups have since been found, up to and includ-

Figure 85. *Streptococcus fecalis.* P strain isolated from focal infection. Smear from pure culture. Fuchsin; × 1050.

Incidence of Streptococci of Groups A and C in Human Infections[21]

TYPE OF INFECTION	NUMBER OF STRAINS	GROUP A		GROUP C	
		NUMBER	PER CENT	NUMBER	PER CENT
Scarlet fever	232	229	98.7	3	1.3
Tonsillitis and septic sore throat	52	52	100.0	0	0
Rheumatic fever and rheumatic arthritis	19	19	100.0	0	0
Puerperal sepsis	55	51	92.7	4	7.3
Erysipelas	51	48	94.1	3	5.9
Miscellaneous	143	136	95.1	7	4.9
Total	552	535	96.9	17	3.1

ing Group N. The biological significance of these groups is indicated by the origin of the strains making them up, *viz.*:

Group A—Primarily pathogens of man
Group B—Found almost entirely in mastitis of cattle
Group C—Primarily pathogens of lower animals
Group D—Found in cheese
Group E—Found in milk

The association of origin and immunological group is, however, not absolute. Streptococci of Group A are occasionally found in lower animals, producing mastitis in cattle for example, while those of Group C are found in man with some frequency as indicated in the accompanying table.

Type specificity among the streptococci was also indicated by the work of Griffith, who separated β-hemolytic streptococci isolated from human disease into twenty-seven types by slide agglutination, and later added three more types. These were given arbitrary Arabic numbers, *viz.*, type 1, type 2, etc. Following definition of the serological groups of Lancefield, it was found that these types are distributed over the groups; Group A included the majority, twenty-three in all, and types 7, 20, and 21 fell into Group C, and type 16 into Group D.

Lancefield has shown that the types within Group A are determined by two type-specific antigens. One of these, the "M" antigen, is a nucleoprotein[52] located apparently very superficially in the cell wall.[3, 4] It is destroyed by proteolytic enzymes, and may be digested by a soluble protease produced by streptococci, resulting in strains which cannot be typed by "M" antiserums. It has been purified to electrophoretic homogeneity but still contains non-type-specific protein antigen.

The other type-specific antigen, designated the "T" antigen, is not as well known biochemically; it is resistant to proteolytic enzymes, but more recent work suggests that it is also protein in nature. The M and T antigens are independent and may occur in various combinations. Thus, types 10 and 12 contain the same M antigen but different T antigens, while types 15, 17, 19, 23, and 30 contain very closely related T antigens but distinct M antigens. In addition, strains are found which lack T antigen, and still others lack, or apparently lack, M antigen. An additional antigen, designated the "R" antigen and similar to M antigen except that it is resistant to tryptic digestion and antiserums to it are not protective, was found first in type 28.[53] Subsequently, it has been found, through use of the indirect bactericidal test,[58] in types 2, 3 and 48.[49, 50]

The nature and distribution of these antigens within the β-hemolytic streptococci of Group A, *i.e.*, *Str. pyogenes*, are shown in the accompanying tables.

While the separation of the streptococci of Group A is a complex matter, streptococcus typing has been rather generally applied, in part because it is of importance in the inter-relationships, possibly phylogenetic, of the streptococci, and in part because it is of great value in epidemiological studies.[75] Many workers feel that slide agglutination is not completely satisfactory and favor the

precipitin reaction; a method of carrying out the precipitin test in capillary tubes which is reliable and conserves serum has been developed.

Content of M antigen varies and is apparently related to the virulence of the strain, while T antigen usually remains constant within a given strain. Since type is determined largely by the M antigen, a strain which has small amounts of this antigen, or has temporarily lost it, may be difficult to type, though type may at times be inferred from the T antigen present. It may be progressively lost during infection coincident with increasing susceptibility to antibody activity,[89] or more than one M antigen may occur as in the type 14 strains, found by Wiley and Wilson[123] to contain a new M antigen, designated 51, and the mixed type as type 14/51; similar multiple type specificity occurs among the pneumococci (Chap. 18). Rarely, the group-specific C polysaccharide may be apparently lost and replaced by a new polysaccharide, V polysaccharide, differing in that the ratios of rhamnose to acetylglucosamine are shifted from 1.6 for the C polysaccharide to 4.5. The change may be a superficial one chemically.[60]

Streptococci falling into other Lancefield groups on the basis of the specificity of the "C" substance are also divisible into types. In Group B, the bovine mastitis streptococci, four main types and a number of subtypes have been differentiated. In this group there is but a single type-specific antigen, and it is polysaccharide in nature. Group C contains, in addition to the three Griffith types noted above, ten additional types, five in strains of human origin, and five in strains from horses, making thirteen in all. Here also there is but a single type-specific antigen, and it is protein in nature. Types have been differentiated in several of the other groups, such as group D,[19] on the basis of a single type-specific antigen, polysaccharide in nature.

While the antigenic structure just discussed is largely that of the β-hemolytic

Distribution of Type-specific Antigens of Streptococcus Pyogenes*

TYPE	M ANTIGENS	T ANTIGENS
1	M 1	T 1 (may be absent)
2	M 2	T 2 (may be absent)
3	M 3	T 3, T 1 (either or both may be absent)
15	M 15	one common T antigen; one common T antigen
17	M 17	
23	M 23	
19	M 19	
30	M 30	
47	M 47	
12	M 12	T 10 and/or T 12 (may be absent)

* Slightly modified from Lancefield: *Streptococcal Infections.* Columbia University Press, 1954, pp. 3–18.

Antigens of Streptococcus Pyogenes*

ANTIGEN	SEROLOGICAL ACTIVITY	CHEMISTRY	
		NATURE	PROPERTIES
C	Group-specific	Polysaccharide	Polymer of N-acetylglucosamine and rhamnose; occurs in cell wall
M	Type-specific	Protein	Alcohol-soluble; resistant to heating in dilute acid; destroyed by proteolytic enzymes: related to virulence, and antibody is protective
T	Occurs in several types but may be type-specific	Protein	Resistant to proteolytic enzymes; unstable to heat in dilute acid, but resistant in slightly alkaline solution
R	Occurs in types 2, 3, 28 and 48 and in strains of Groups B, C, and G	Protein	Destroyed by peptic but not by tryptic digestion; unstable to heat in dilute acid, but stable to heat in dilute alkali

* Modified from Lancefield: *Streptococcal Infections.* Columbia University Press, 1954, pp. 3–18.

streptococci, β-hemolysis is not invariably associated with the immunological character of these groups, and it has been found that certain of the enterococci, for example, contain these antigens. By and large, however, this antigenic structure does not extend far beyond the β-hemolytic group, and, as indicated above, immunological methods have not been useful in other than this group.

Physiological differentiation. There is some correlation between the immunological groups of the hemolytic streptococci and their physiological characteristics. Group A shows a reasonable degree of biochemical homogeneity. All produce β-hemolysis on blood agar and

I. Facultative anaerobes
  A. Pyogenic group
    1. Sodium hippurate not hydrolyzed
      a. Lactose fermented
        i. Sorbitol –, trehalose + — Lancefield Group A
          Streptococcus pyogenes
        ii. Sorbitol +, trehalose – — Lancefield Group C
          Streptococcus zooepidemicus (animal pyogenes)
      b. Lactose fermentation variable
        i. Trehalose –
          Streptococcus equi
        ii. Trehalose +
          Streptococcus equisimilis
    2. Sodium hippurate hydrolyzed — Lancefield Group B
      Streptococcus agalactiae (*mastitidis*)
  B. Viridans group
    1. Lactose fermented
      a. Does not grow at 50° C.
        i. Starch not hydrolyzed, not tolerant of bile
          Streptococcus salivarius
          Streptococcus mitis
        ii. Starch is hydrolyzed, bile-tolerant
          Streptococcus bovis
      b. Grows at 50° C.
        Streptococcus thermophilus
    2. Lactose not fermented
      Streptococcus equinus
  C. Lactic group
    1. Maltose +, dextrin +, ammonia from peptone
      Streptococcus lactis
    2. Maltose –, dextrin – (usually), no ammonia from peptone
      Streptococcus cremoris
  D. Enterococcus group — Lancefield Group D
    1. Not β-hemolytic
      a. Does not hydrolyze gelatin
        Streptococcus fecalis
      b. Gelatin hydrolyzed
        Streptococcus liquefaciens
    2. β-hemolytic
      a. Mannitol +, sorbitol +
        Streptococcus zymogenes
      b. Mannitol –, sorbitol –
        Streptococcus durans

II. Micro-aerophilic or obligate anaerobes
  Streptococcus anaerobius
  Streptococcus foetidus
  Streptococcus putridus
  Streptococcus lanceolatus
  Streptococcus micros
  Streptococcus parvulus
  Streptococcus intermedius
  Streptococcus evolutus

form soluble hemolysins, do not hydrolyze sodium hippurate, ferment trehalose but not sorbitol, do not reduce methylene blue, and seldom grow in 40 per cent bile agar. Group B is also more or less homogeneous, differing from group A in that sodium hippurate is hydrolyzed and growth occurs in 40 per cent bile agar, but resembling that group with respect to methylene blue reduction and trehalose and sorbitol fermentations.

The streptococci of Group C are considerably more diverse and resemble Group A in that sodium hippurate is not hydrolyzed. Within the group there is some correlation between habitat and physiological character. Thus the strains isolated from strangles in horses are sorbitol-, trehalose- and lactose-negative, while strains that are pathogenic for man generally ferment sorbitol but not trehalose.

Physiological characteristics have been used exclusively in the Bergey (1957) classification of the streptococci and the species differentiated on that basis are indicated in the accompanying abridged key (p. 488).

Most of these species are generally accepted, and it will be clear from the foregoing discussion that all members of group A are known as *Str. pyogenes*, the immunological varieties within the group being types of *Str. pyogenes*. Two species are included in Group C. The name *Str. zooepidemicus* is a new one introduced in the Bergey (1948) classification; it does not replace another name, but rather gives species status to the animal pathogens of this group formerly casually known as "animal pyogenes" which rarely if ever occur in man. The other species, *Str. equisimilis*, includes those streptococci formerly known as "human C" and which are uncommon in lower animals. Of the β-hemolytic streptococci found in man, 95 per cent or more are Group A and therefore *Str. pyogenes*, and the remainder, Group C, are *Str. equisimilis*, differentiation being made by the precipitin test, using group-specific antiserums. Of the green streptococci found in man, the most common are *Str. fecalis* of the enterococcus group and *Str. mitis* and *Str. salivarius* of the viridans group. So far as the etiology of streptococcal infections in man is concerned this differentiation and that of *Str. fecalis* and *Str. liquefaciens* are unimportant; in fact it is open to serious question as to whether the last should be regarded as other than a variant of *Str. fecalis*. The identification of streptococci which do not fall into the Lancefield groups is a matter of detailed biochemical study.

Pathogenicity for Animals. As indicated above, certain of the streptococci are responsible for specific diseases of domestic animals. *Str. equi* is the cause of strangles in horses, a suppurative infection of the upper respiratory tract that is characterized by abscess formation in the throat and submaxillary region. This species is apparently not pathogenic for man. Other strains of Group C infect horses, causing respiratory catarrh and suppurative lesions in various parts of the body. The most common cause of streptococcal mastitis in the cow is *Str. agalactiae* which produces a chronic infection that is, as a rule, more common in older cattle. The infection is probably spread by the hands of the milker for the most part. Mastitis may also be caused by *Str. pyogenes* and may result in the spread of milk-borne streptococcal infection of man; it is probable that the source of infection is man and that *Str. pyogenes* does not occur naturally in the cow. Various other suppurative conditions, often involving infection of lymphatic tissue in animals such as the dog and sheep, are of streptococcal etiology. Some are Group A streptococcal infections, as in the case of epizootics which have been observed to occur in wild rodents[6] and laboratory mice,[37] but usually are β-hemolytic streptococci of groups other than Group A; there are a few reports of spontaneous infections of lower animals with green streptococci.

Str. pyogenes is pathogenic for most laboratory animals, including the mouse, guinea pig, and rabbit, but different strains vary widely in virulence for different animals; virulence may, of course,

be enhanced by animal passage. Intravenous inoculation of virulent strains results in fatal septicemia; suppurative peritonitis developing into septicemia follows intraperitoneal inoculation, and subcutaneous inoculation produces an abscess from which the infection may or may not spread. Pneumonia may be produced in rats by the intrabronchial inoculation of the bacteria suspended in mucin.[29] *Str. agalactiae* is of low virulence for experimental animals, and *Str. equi* is highly virulent only for the mouse. The green streptococci are also relatively avirulent for laboratory animals but may produce local infections on intravenous inoculation following injury; such techniques have been used to produce experimental arthritis lesions in the rabbit.

Pathogenicity for Man.[59] The streptococci are responsible for a wide variety of diseases of man, perhaps a greater variety than any other kind of bacteria, and, in addition to being the primary cause of disease, they have a marked tendency to occur in mixed and secondary infections with other pathogenic bacteria. In general, the streptococcal infections are characterized by suppurative lesions and very often manifestations of toxemia, the latter taking the form of the so-called nonsuppurative complications of the infection and including fever, arthritis, carditis, and nephritis. They vary in the extent to which the body is involved, from local infections such as abscesses of the various tissues, including mucous membranes, joints, and serous membranes, infection of the muscle or cellulitis which simulate gaseous gangrene, suppurative processes in all kinds of wounds, to those generalizing to pyemia or septicemia. Some streptococcal infections have no distinctive clinical manifestations; thus an abscess caused by streptococci is not distinguishable from one of staphylococcal etiology. Others, however, such as erysipelas, streptococcus sore throat, scarlet fever, and the like, have to a greater or lesser degree some distinctive clinical character.

There are appreciable differences in the character of streptococcal infection in the child and in the adult, and the lower age groups are relatively the more susceptible. In the infant the infection tends to be a prolonged, low-grade infection with frequent suppurative complications, but rheumatic fever and nephritis seldom follow. In older children and adults the infection tends to be acute and self-limited and with nonsuppurative complications.

The β-hemolytic streptococci are by far the most virulent and, as indicated above, the great majority of those infecting man are of Group A, *Str. pyogenes.* The small proportion of infections with Group C streptococci are not distinguishable from those of Group A except by isolation and immunological typing of the etiological agent.

The pathogenicity of these bacteria is accounted for to a considerable degree by the soluble toxic substances they produce.[2] The most clear-cut example is that of the relation of the erythrogenic toxin to scarlet fever, but it is probable that other toxic substances also play a part in the development of the pathological condition.

The association of fibrinolysin with virulence seems definite, so much so that this activity has been termed invasin by some workers.

The role of the streptolysins in the development of the pathology of streptococcus infections is not too clear. Both streptolysin S and streptolysin O are toxic for experimental animals, the former causing death through intravascular hemolysis. The mechanism of the lethal action of streptolysin O is not known; it may possibly be related to the cardiotoxic action of this substance described by Bernheimer. There is some reason to believe that the destructive effect of streptococcal filtrates on polymorphonuclear leucocytes, attributed to the presence of a leucocidin, is closely related to or identical with streptolysin O, but in any case this activity may make up a significant part of the invasive and pathogenic properties of the streptococci. The presence of the type-specific M antigen is associated with virulence,

and antibody to it is protective while antibody to the type-specific T antigen is not. Hyaluronic acid capsules seem to be at best a minor component of virulence among Group A streptococci but appear considerably more important in this respect in Group C.

In general, then, while knowledge of the mechanisms of the pathogenic action of the β-hemolytic streptococci is far from complete, there is a considerable body of evidence which indicates that the production of disease is due at least in part to the action of the several toxic substances formed by these bacteria. As indicated earlier, strains of streptococci differ with respect to the kinds and amounts of such substances that they produce, and this variability accounts in part for the differences in disease they may produce. Thus, both strains which produce erythrogenic toxin and those which do not may produce septic sore throat, but only the former can produce scarlet fever also. These are not the only factors, of course, and the route of infection and immunity of the host are significant also; for example, wound infection and streptococcal sore throat involve different routes of infection, and an erythrogenic toxin-producing strain can produce sore throat but not scarlet fever in the immune.

Streptococci other than the β-hemolytic varieties are much less virulent.[22] The α-hemolytic forms constitute a portion of the normal bacterial flora of the mouth, upper respiratory tract, and intestinal tract. It is probable that they seldom initiate infection of the healthy tissues, but when natural resistance has been reduced they may be able to set up low-grade, essentially localized, infections such as focal abscesses in the teeth and gums.[88] They are the most common cause of subacute bacterial endocarditis but, while the condition is a serious one, the infection shows little or no tendency to spread throughout the body in spite of the frequent presence of the streptococci in the blood stream.

Pneumonias of α-streptococcal etiology occur from time to time.[20, 74] The disease tends to assume a chronic relapsing condition, with pleuritis and lung infiltration, and a tendency to bilateral involvement. It resembles viral pneumonias superficially, but differs in that there is a marked leucocytosis. A few instances of α-hemolytic streptococcal meningitis have been reported,[38] but are rare.

Streptococcus MG. The nonhemolytic, or anhemolytic, streptococci are largely saprophytic forms, often found in milk and dairy products, and have been associated with disease only in rare cases of subacute bacterial endocarditis. They are occasionally isolated in atypical pneumonias, which are most often of varied viral etiology, but apparently are not causally related to the disease. One such strain, designated streptococcus MG, has been isolated from the sputum in a series of cases of primary atypical pneumonia, and proved to be biochemically and antigenically homogeneous.[64, 65] About half the cases of such disease develop specific agglutinins for this strain of streptococcus, and these are known as MG agglutinins. This antibody response is distinct from the cold hemagglutinin that also occurs in primary atypical pneumonia, and the two kinds of antibody have empirical diagnostic utility although so far as is known they have no bearing on the etiology of this disease[24, 25, 112] (Chap. 38).

Epidemiology of Streptococcal Disease. The primary source of pathogenic streptococci is the human being who carries these bacteria in the upper respiratory tract. The infection may not be associated with symptoms, and carrier rates of 4 to 25 per cent to as much as 40 to 60 per cent in school children, with cumulative percentages as high as 75 to 90 per cent,[77] have been reported. Those with overt symptoms of disease such as tonsillitis, pharyngitis, sinusitis, and scarlet fever are prolific sources of infection. While streptococci may be present in the saliva as well as in the throat, and discharged by sneezing, coughing, and contamination of the hands, the nasal carrier is by far the most dangerous and contributes very large numbers of streptococci to his environment. Not only is the carrier the

source of infection, but the streptococcal disease may take a variety of clinical forms. In an epidemic of scarlet fever, for example, the cases of pharyngitis and rhinitis are as important as those of frank scarlet fever, *i.e.*, those showing a rash, in the spread of the infection; in fact it is customary to record the incidence of scarlatinal rash in a given epidemic rather than differentiate scarlet fever from other streptococcal infections of the upper respiratory tract.

The transmission of streptococci from the infected person to the susceptible individual is in part a matter of direct contact and in part one of contamination of the environment.[55, 72, 73] Direct contact may include inhalation of infective droplets expelled from the nose and mouth, hand-to-hand contact, etc., while contamination of the environment is the contamination of the air with droplets too small to settle, and through air and droplet infection the contamination of dust. Direct contact with the hands no doubt accounts for wound infection, puerperal fever, infection of the udder with *Str. pyogenes* to produce mastitis and milk-borne streptococcal disease, and possibly infection of the upper respiratory tract to a certain extent. Most upper respiratory tract infection is airborne,[33, 34] either directly or through the agency of resuspended infected dust. The importance of the last is very great, and dust suppression measures such as oiling of blankets in hospital wards sharply reduce the incidence of streptococcal infection.

Although some streptococcal diseases are reportable, the incidence of streptococcal infection can only be estimated; probably about seven million infections occur each year in this country.

Immunity to Streptococcal Infection. Antibodies to the streptococcus cell substance and to the antigenic soluble products of these organisms are formed following immunization or infection. In the case of *Str. pyogenes*, the former include the type-specific M and T antigens as well as the group-specific C substance. In the latter group are the erythrogenic toxin, streptolysins S and O, hyaluronidase, and fibrinolysin. Antibody formation has no diagnostic utility in acute streptococcal infections, in part because there is not sufficient time for antibody formation during the course of the disease, at least in its early stages, and in part because isolation and, if desirable, typing of the infecting microorganism are relatively simple. Antibody titration is useful for diagnosis in retrospect, *i.e.*, in associating streptococci with diseases such as rheumatic fever and arthritis, and for the determination of susceptibility to scarlet fever on the basis of antibody to the erythrogenic toxin.

The immunized animal responds to the cell antigens of streptococci with the production of precipitating and agglutinating antibody, and the use of such antiserums makes possible immunological grouping and typing. The immune response of man to these antigens during the course of infection appears more often to take the form of the development of a hypersensitivity, especially in the rheumatic diseases, and the immunological response may be measured by intradermal inoculation of soluble streptococcal antigens. More often serum is titrated for antibody to streptolysin or fibrinolysin by *in vitro* methods. Titration of antibody to streptolysin O may require caution in interpretation in that human serum may in some conditions, especially hepatitis, have an inhibitory activity associated with lipid content and unrelated to antibody.

Quite aside from the question of the etiology of such diseases, the occurrence of such antibodies in the human population is not uncommon, as might well be expected from the frequency with which streptococcal infection occurs. It is sometimes stated that 70 to 80 per cent of persons having streptococcal disease show significant increases in antibody titer to streptolysin O, streptokinase, and hyaluronidase. The erythrogenic toxin also stimulates the formation of specific antitoxin, following both clinical scarlet fever and immunization with the toxin, and, like diphtheria antitoxin, the incidence of its occurrence rises with age, indicating that immunization occurs

without the intervention of clinical scarlet fever. Intradermal inoculation of erythrogenic toxin gives a skin test, analogous to the Schick test in diphtheria, which allows the estimation of the immunity of the individual to the toxin. Antibody to the erythrogenic toxin cannot be titrated by *in vitro* methods, or to a satisfactory degree in experimental animals because of their relative lack of susceptibility to its action.

Differentiation must be made between an immune response in the technical sense of antibody formation and an effective immunity which prevents and/or modifies the infection or disease. The only immunity to streptococcal disease that is unquestionably effective is immunity to the erythrogenic toxin, which is reflected as immunity to the clinical disease scarlet fever. In general immunity to streptococcus infections is of a low order and in any case transient. That some degree of effective immunity to a given type of streptococcus may be produced is indicated by recovery from and elimination of the infection in naturally occurring disease, and there is also some experimental evidence of the existence of an effective immunity. Watson, Rothbard, and Swift,[119] for instance, have shown that a nasopharyngeal carrier state induced in monkeys resulted in an increase in antistreptolysin O titer of the serum and a resistance to re-implantation with the same strain that persisted for some months.

Unfortunately such immunity appears to be type-specific and, as pointed out earlier, associated with antibody to the M antigen, and there is only a small degree of cross-immunity. Since the M antigen appears to be located primarily, or even exclusively, in the cell wall, it has been inferred by some that its association with virulence is analogous to that of the pneumococcus capsule in that it interferes with phagocytosis. It would follow, then, that the effective immune response is opsonic, and studies utilizing the phagocytic index and similar criteria show a correlation between effective immunity and this humoral antibody. With the multiplicity of streptococcus types in the species *Str. pyogenes*, an effective immunity to streptococcus infection appears to be a somewhat impractical end.

Chemotherapy. The chemotherapeutic efficacy of the sulfonamides was first observed in experimental infections with *Str. pyogenes*. These compounds continue to be effective chemotherapeutic agents in infections with the streptococci but have been displaced to a considerable extent by the antibiotics, especially penicillin. Penicillin has been of special interest in the mass prophylaxis of epidemic streptococcal infection and in reducing the carrier rate in military populations.[96, 97, 98] The hemolytic streptococci are more susceptible to these agents *in vitro* than are the staphylococci. The susceptibility of the green streptococci to the antibiotics is, in general, approximately the same as that of the staphylococci, but is subject to such wide variation from strain to strain that antibiotic sensitivity tests are practically mandatory. Bacterial endocarditis of α-hemolytic streptococcal etiology is the most important of the diseases produced by these streptococci, and the susceptibility of the causative strain is usually such that massive therapy is effective.[57]

The relative efficacy of chemotherapy is dependent upon the kind of disease produced by the streptococci. For example, puerperal sepsis, streptococcal sore throat, pneumonia, and erysipelas generally respond well to chemotherapy, but the rash of scarlet fever is not affected, nor is the therapy of acute rheumatic fever satisfactory. Chemotherapy does eliminate the focus of infection in scarlet fever and decreases the incidence of suppurative complications. The failure of rheumatic fever to respond to antimicrobial therapy is due to the lag of symptoms behind the actual infection and probable role of hypersensitivity (see below). Prevention of recurrent streptococcal infection in affected individuals is highly significant, and repository penicillin is an effective prophylactic agent.[107]

As in certain other infections, the prompt use of effective chemothera-

peutic agents, especially penicillin in streptococcal infections, inhibits the immune response as compared to the untreated infection, probably as a consequence of reduction in the total antigenic stimulus.

Bacteriological Diagnosis of Streptococcal Infection.[124] Isolation of streptococci from specimens of pathological material is ordinarily not difficult. An enriched medium is required, and blood agar is the medium of choice, for both α-hemolysis and β-hemolysis are apparent. Overgrowth by Proteus in cultures of some kinds of specimens may be prevented by including 0.02 per cent sodium azide in the medium. Most specimens, such as throat swabs and pus, may be streaked directly on blood agar, but enrichment culture, as in veal infusion broth containing 0.1 per cent dextrose and 0.1 per cent phosphate buffer, should be made with blood taken for culture, incubated for 24 hours, and then streaked on blood agar. If there is reason to believe that the specimen contains sulfa drug, its bacteriostatic effect may be neutralized by including 5 mg. per 100 ml. of *p*-aminobenzoic acid in the medium.

The colonial morphology is typical in the case of β-hemolytic streptococci, and the characteristic chains of cocci may be found in Gram-stained smears. Green streptococci from sputum and similar specimens must be differentiated from pneumococci by inulin fermentation and bile solubility. The hemolytic streptococci may be typed by agglutination with type-specific antiserums and by the precipitin test. For the latter the sedimented bacteria from a 250 ml. broth culture are suspended in 10 ml. of M/10 HCl in saline, boiled for 10 minutes, cooled in running water, and the insoluble material spun out to leave a clear supernatant to be used as the antigen. Considerable economy of reagents may be effected by setting up the precipitin test as a ring test in capillary pipettes; the precipitate at the serum-antigen interface may be observed with a hand lens.

STREPTOCOCCAL INFECTION OF THE SKIN AND SUBCUTANEOUS TISSUES

Erysipelas. The ability of streptococci to infect the skin and adjacent tissues is well illustrated in erysipelas, an inflammatory disease of the skin caused by *Str. pyogenes*. There is some evidence that an attack of the disease is preceded by streptococcal infection of the throat or elsewhere in the upper respiratory tract, and it has been found that some individuals at least have the same immunological type of streptococcus in the throat as in the skin lesions. It is not clear whether the skin is directly invaded, or whether the microorganisms reach the area by some internal route, but the latter is only suggested and by no means established. The etiological relationship of *Str. pyogenes* to the disease is indicated by its presence, frequently in enormous numbers, in the lesions, the production of erysipelas-like disease in rabbits by the inoculation of streptococci, and inoculation experiments in carcinomatous patients which have demonstrated that pure cultures of streptococci can provoke the erysipelatous process. It is possible that hypersensitivity may contribute to the pathogenesis of the disease.[5]

Streptococci are not present in the central portion of the inflamed area, but are found on its periphery, and can be isolated most readily by excision of portions of the tissue, other methods rarely succeeding. In the skin they occur chiefly in the lymph spaces, which are often packed with them, and may be recovered by skin puncture as far as 3 cm. beyond the advancing edge of the lesion where there is no gross evidence of inflammation. The hypothesis that the inflammatory reaction is due in part at least to the erythrogenic toxin has been an attractive one, but it seems definitely established that there is no relation; immunization with erythrogenic toxin, for instance, in no way prevents or reduces the inflammatory reaction in erysipelas.

This disease, and especially experi-

mental erysipelas in the rabbit, has been of considerable interest in connection with problems of local and tissue immunity. It is well established that one attack of the disease confers no protection against subsequent attacks, and in the opinion of many, some persons have a predisposition to the disease, suffering repeated attacks throughout life and even in the same areas. In the experimental disease in the rabbit, however, a number of workers have reported that successive intradermal inoculations bring about an increased resistance to subsequent inoculation in that area, and that with continued immunization the area increases slowly in size. The immunity is not highly specific, and inoculation of sterile broth also results in some increase in resistance to infection. Consistent with this, immune serum, when mixed with streptococci and inoculated intradermally, seems to have some protective effect. Serum therapy of the human disease, however, appears to produce only a possible mildly favorable effect upon the immediate attack but none upon recurrences and complications such as abscess formation.

Wound Infection.[76] The green streptococci seldom occur in infections of wounds, but *Str. pyogenes* produces a suppurative infection when present, especially late. This organism is not normally present on the skin, and, in fact, normal skin has a bactericidal effect upon it. The relative rarity of infection of wounds by these bacteria is consistent with this, and streptococcal infection in most instances is a result of subsequent contamination by direct contact rather than of a primary infection. *Str. pyogenes* may occur alone or in mixed infections with other pyogenic bacteria such as staphylococci.

Cellulitis.[56] Traumatic invasion of the skin and subcutaneous tissues may not remain localized but may develop into an acute, spreading infection of the subcutaneous tissue with invasion of the muscle giving rise to gangrenous myositis. The infection of the subcutaneous tissues may show little or no evidence of localization and is characterized by the formation of a seropurulent exudate. It tends to spread rapidly via the lymphatic tissues and generalize into septicemia. This kind of streptococcal infection has been termed cellulitis, and may result from *Str. pyogenes* alone or, in the development of the gangrenous process, more often in mixed infection with anaerobic streptococci. This kind of wound infection was found in World War II.

STREPTOCOCCAL INFECTION OF THE UPPER RESPIRATORY TRACT[12]

As indicated earlier, the β-hemolytic streptococci occur most commonly as parasites and pathogens of the upper respiratory tract. By far the largest proportion of human disease caused by *Str. pyogenes* results from infection of the upper respiratory tract and adjacent areas, symptoms arising not only from the acute infectious process, but also in connection with its complications. The clinical character of the disease is determined by the relative prominence of the various results of the infection and, while seemingly different, is fundamentally the same Thus streptococcal pharyngitis or septic sore throat becomes scarlet fever when the infecting strain of *Str. pyogenes* produces erythrogenic toxin, the infection commonly extends into the tonsils or may be localized primarily there to give clinical tonsillitis; it may extend into the sinuses or middle ear to produce streptococcal sinusitis and otitis media respectively, and by extension into the lungs result in bronchopneumonia of streptococcal etiology. Further, late nonsuppurative complications of streptococcal infection include carditis, nephritis, and arthritis. While separation of β-hemolytic streptococcal infection into various clinical entities has some practical value, the basic infectious process is essentially the same.

The green streptococci are also inhabitants of the upper respiratory tract, as pointed out earlier, and are so constantly present and of such restricted

pathogenicity that they are a part of the normal bacterial flora. Infection of the teeth and gums undoubtedly stems from this area, and it is likely that the bacteria entering the blood stream to produce local infections elsewhere in the body originated in the upper respiratory tract. They are occasionally found in bronchopneumonia.[105, 111]

Streptococcus Sore Throat (Septic Sore Throat). The β-hemolytic streptococci are responsible for an acute infection of the throat commonly known as septic or streptococcus sore throat. Epidemics of this disease appeared in the United States and in England during the first decade of the present century. The immediate symptoms in these and subsequent epidemics have been strikingly similar and include an intense local hyperemia, with or without a grayish exudate, enlargement of the cervical lymph nodes, and usually fever. Extension of the infection into the lungs may occur with resulting streptococcus pneumonia[40] which may terminate in fatal septicemia, and peritonitis has been a cause of death also. The disease, usually in a relatively mild form, is a common one.

The sequelae of streptococcal sore throat include those resulting from the extension of the infection into adjacent areas such as the sinuses and middle ear, and purulent, semichronic infections often develop. Streptococci also frequently persist in tonsillar crypts in a chronic type of infection which may flare up periodically in an acute form. Streptococcal tonsillitis, sinusitis, and otitis media are, then, a part of the pathology of hemolytic streptococcus infection of the throat. In addition to such extensions, the effects of toxemia on other parts of the body are evident as carditis, nephritis, and arthritic involvement of the joints. As in the case of scarlatinal rash noted earlier, the character of an epidemic is often recorded as percentage incidence of these various sequelae.

The distinctive clinical character and epidemic spread of the disease led earlier workers to believe that it was caused by a particular kind of streptococcus to which the name *Str. epidemicus* was given. It is now established that various immunological types of *Str. pyogenes* are, for the most part, responsible for the disease, and a small portion of the cases are infections with streptococci of Group C, now grouped as *Str. equisimilis*. On the other hand, there is reason to believe that so-called "epidemic strains," of high virulence and in-

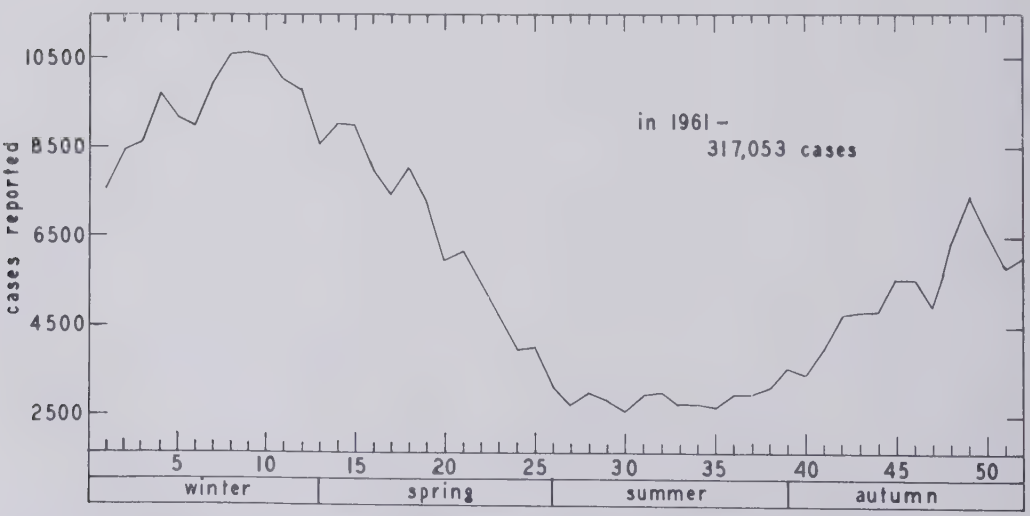


Figure 86. The seasonal incidence of streptococcal infection as illustrated by the numbers of cases reported weekly in 1961. Data from the Morbidity and Mortality Weekly Report, Communicable Disease Center, Public Health Service, U. S. Department of Health, Education and Welfare.

fectivity, of streptococci as well as other bacteria are often associated with epidemic disease.

It is probable that the infection is largely droplet and airborne, including dust, but there is no doubt that in many instances direct contact is of considerable significance. It may also be transmitted through food and milk and streptococcal infection is often milk-borne. In the past it has been assumed that the contamination was direct from man, but evidence has accumulated which indicates that mastitis of *Str. pyogenes* etiology may constitute the immediate source of infection of the milk.

SCARLET FEVER

Scarlet fever is a clinical entity because of the rash resulting from the action of the erythrogenic toxin; otherwise it does not differ significantly from other streptococcus infection of the upper respiratory tract, and its sequelae are essentially the same.

While scarlet fever has declined in prevalence and severity since the turn of the century, following a period of high prevalence during the nineteenth century, it has behaved differently in different parts of the world in the last two or three decades. It increased in continental Europe after World War II and remained at a high prevalence, while in England and Wales it continued at a fairly constant level, and has declined sharply in the same period in the United States and Canada.[71, 131, 132]

The relationship of β-hemolytic streptococci to the disease was demonstrated by Dick and Dick in 1923 by the reproduction of typical scarlet fever in human volunteers, by the inoculation of pure cultures, and by the demonstration of the existence of the erythrogenic toxin. A conclusive demonstration of the etiological relation was required because of the contrast between the relatively lasting immunity to scarlet fever following recovery from an attack of the disease and the transient immunity to other streptococcal infections. It has been held by the Dicks and others, largely on the basis of early agglutination studies, that the scarlet fever streptococci constitute a homogeneous group of β-hemolytic streptococci which should be designated *Str. scarlatinae*. While the streptococci found in scarlet fever are all members of Group A, *i.e., Str. pyogenes,* the ability to form erythrogenic toxin is not confined to any particular type within this group, though some types occur more frequently than others, and it cannot be said that the scarlet fever streptococci are immunologically homogeneous.[26, 32] Neither are they biochemically homogeneous, and in the Dicks' early experiments the mannitol fermentation was variable in scarlet fever–producing strains. It appears, then, that the scarlet fever streptococci are strains of *Str. pyogenes* that have in common the ability to form erythrogenic toxin, but such strains are found in conditions other than scarlet fever, such as erysipelas.

The Erythrogenic Toxin.[18] It is generally agreed that the scarlatinal toxin is protein in nature, but some workers have reported that it is heat-stable and others that it is heat-labile. It has been found, however, that heat-lability is determined by methods of preparation. The purest preparations, prepared by ammonium sulfate and cold ethanol fractionation, contain 11,250 Lf/mg. N or 2100 Lf/mg. protein. Four components have been demonstrated by electrophoresis; the toxicity was associated with the slowest, and on separation this contained 10^8 STD per mg. Whatever its nature, it is necessarily assayed by skin test and its potency is measured in skin test doses (STD), the smallest amount that will produce the characteristic erythematous response. It is antigenic and is floccul ted by antitoxic serums but in multiple zones, and the flocculation titration must be carefully controlled.[41]

Scarlet Fever Antitoxin. The therapeutic use of antistreptococcus serums was investigated by Moser in 1902 with encouraging results, and similar observations were reported by Dochez and others in 1924. The Dicks prepared spe-

cific antitoxic serums by immunizing horses with sterile culture filtrates. The results of the therapeutic use of the Dick antitoxin are favorable on the whole, though the mildness of the prevalent type of scarlet fever makes it difficult to secure any large body of statistics as cogent as those recorded in diphtheria. In individual cases, however, the administration of antitoxin decreases the duration of the rash, changes the character and extent of desquamation, and reduces the number of complications, and there is general agreement as to the efficacy of antitoxic serum properly prepared and administered early. Some clinicians would restrict the use of scarlet fever antitoxin to cases of severe or toxemic type. A unit of antitoxin is defined as that amount which will neutralize 50 skin test doses of toxin.[80]

Immunity to scarlet fever is, in a sense, a clinical immunity in that it is largely an immunity to the erythrogenic toxin rather than to the streptococcus. It may be demonstrated by the *Dick test,* a skin test analogous to the Schick test in diphtheria, *i.e.,* the local erythema is due to the action of the toxin and is absent in the presence of antitoxin, either of exogenous origin or present in the immune individual. The Dick test may be used, then, to ascertain whether or not an individual is immune to scarlet fever or, more precisely, whether circulating antitoxin is present. In this connection it is of interest that Schultz and Charlton earlier observed that when a scarlet fever patient with a bright-red rash is injected with 1 ml. of convalescent serum, after about six hours the rash begins to fade and soon disappears completely. The significance of this phenomenon, the *Schultz-Charlton blanching phenomenon,* was not recognized at the time.

Scarlet fever, like the other hemolytic streptococcal infections, is effectively treated with penicillin, to reduce the period of illness and the number of complications. Early chemotherapy seems to interfere with the development of the immune response, as indicated by the antistreptolysin titers, and the relapse rate is not reduced.[99]

Prophylactic Inoculation.[86] It is not generally known that following Jenner's work on smallpox vaccination attempts were made to immunize against scarlet fever by a similar process of inoculation. Preventive inoculation with killed streptococcus cultures was practiced by Russian bacteriologists as early as 1906. Mild symptoms were produced similar to those observed in scarlet fever. A single injection did not suffice to produce immunity, two or three inoculations being necessary. It was believed that a considerable degree of immunity was obtained by this procedure.

The discovery of the scarlet fever toxin offered an opportunity for protective immunization similar to that successfully utilized in diphtheria. Toxins with a potency of at least 40,000 STD per cc. are preferable, and appropriate dilutions are injected at intervals of one week. Five injections are recommended, starting with 500 STD and gradually rising to about 100,000 STD. Immunization of susceptibles (Dick-positives) in this manner produces 98 per cent or more Dick negatives. The injections are sometimes accompanied by the development of a scarlatiniform rash and other symptoms of mild scarlet fever. In consequence, the use of toxin detoxified by treatment with formalin has been advocated by some workers. It is to be emphasized that immunity to scarlet fever is an immunity to clinical scarlet fever, not to the streptococcal infection, and from the epidemiological point of view both Dick-negative and Dick-positive individuals must be considered as having scarlatinal infections, the only difference being the clinical one dependent upon the development of a rash.

As in the case of diphtheria, immunity to scarlet fever may be acquired by inapparent infection. The frequency of positive Dick tests is low in newborn children (indicating a passive immunity of maternal origin), then rises to a maximum in the age groups 1 to 5 years, and thereafter falls off gradually until in

persons over 30 it is relatively low, possibly 15 per cent.

RHEUMATIC FEVER[31, 45, 48, 104,109]

As indicated earlier, not uncommon sequelae of β-hemolytic streptococcus infection include carditis and arthritic involvement of the joints. These are emphasized and assume a major role in the pathology and symptomatology of the post-streptococcal nonsuppurative inflammatory disease known as rheumatic fever, acute rheumatism, or rheumatic heart disease and occurs as a sequel to streptococcal infection in about 3 per cent of cases. The essential lesion of rheumatic fever is carditis which may or may not be accompanied by pyrexia and arthritis. The carditis includes the connective tissue degeneration characteristic of the damaged heart valves, and specific inflammatory myocardial lesions characterized histologically by nodular collections of cells described by Aschoff and known as Aschoff nodules. Rheumatic fever is the third most common infectious disease in this country, exceeded only by tuberculosis and syphilis.

The connection between β-hemolytic streptococcus infection and rheumatic fever is very close, and there is good reason to believe, though as yet not complete and unequivocal proof, that the relationship is etiological. The epidemiological association of streptococcal infections such as tonsillitis, puerperal fever, scarlet fever, and the like, with rheumatic disease has long been recognized, and is reinforced by evidence of an immunological response, such as antistreptolysin O, to streptococcal infection almost invariably associated with rheumatism.

Rheumatic carditis occurs in some individuals; the lesion is a nonpurulent inflammation of the valve cusps and rings and formation of verrucae on the margins which are eventually replaced with scar tissue. Rheumatic myocarditis commonly occurs also, and is characterized histologically by the perivascular granulomata known as Aschoff bodies or nodules.[115] Some observations suggest that there is an individual tendency to valvular involvement, and when this does not occur in the first attack, it is less likely to in subsequent attacks.[23]

The onset of the disease does not necessarily coincide with acute streptococcal disease and only rarely has it been possible to culture β-hemolytic streptococci from the blood. Largely through the work of Coburn,[10] beginning in 1931, these discrepancies have been reconciled, and the etiological relationships of the β-hemolytic streptococci clearly indicated.

Coburn differentiates three stages in the disease: first, an acute streptococcal infection of the nasopharynx; second, a period of quiescence during which streptococci remain in the throat; and third, the stage in which electrocardiographic changes are apparent, and the symptoms of acute rheumatic fever appear. During the third stage streptococci may or may not be demonstrable, and the symptom complex appears to be a consequence of a developed hypersensitivity to the streptococcal antigens (see below). The time which elapses between the first and third stages varies from one to five weeks in patients in whom it has been studied. A practically significant point is that, since clinical rheumatic fever is not a direct result of streptococcal activity, chemotherapy is not effective.

Subsequent attacks occur on reinfection with streptococci, or occasionally following nonspecific febrile events, and control of the disease in the individual consists in chemoprophylaxis.[66, 85, 121] Repository penicillin, *e.g.*, benzathine penicillin G, and sulfonamides are satisfactory, but the broad-spectrum antibiotics are not.[9] The time over which prophylaxis must be continued is uncertain, but some evidence suggests that recurrences drop sharply after age eighteen.[43]

The mechanism by which streptococci produce rheumatic fever is far from clear. The antibody response to the initial infection is delayed in that peak

titers of antistreptolysin O, complement-fixing antibody, and group and type-specific precipitins occur in the third stage of the disease rather than earlier, while antistreptolysin S declines in the third stage, particularly in those having recrudescences. There is reason to believe that hypersensitivity exists to the streptococcus or its products; for instance, joint pains are often produced in rheumatic patients by the inoculation of sterile streptococcus culture filtrates. It appears that an immune response is basic to these consequences of streptococcal infection, but whether this is a conventional antibody response or a hypersensitivity, or both, is not clear. As regards the former, there is reason to suspect that an auto-immune response occurs[44] which would put rheumatic fever and its sequelae with the collagen diseases; there is, for example, evidence of bound γ-globulin in cardiac tissue. A sensitization may be one to streptococcal antigen, and if so it is an antigenicity widely distributed over types of *Str. pyogenes,* or to a complex of streptococcal and tissue antigenicities.

It is quite likely also that the role of the host is of considerable importance. There is evidence of hereditary susceptibility to rheumatic fever,[130] and some indication of a constitutional predisposition to rheumatic fever; the disease occurs more commonly in hyperthyroid persons, there is some evidence suggesting that lipid metabolism may be involved, and it occurs more commonly in the 5- to 15-year age group than in older persons.

It has been of some interest that many of the lesions characteristic of rheumatic fever may be produced in the rabbit by inoculation of β-hemolytic streptococci. These include nonsuppurative myocarditis (without, however, the typical Aschoff nodule), endocarditis, and arthritis ranging from mild to severe with extensive changes in the bone.[67] While it may be argued that such pathology is not identical in all respects with that of rheumatic fever in man, the fact that such lesions can be produced experimentally by the inoculation of streptococci may be regarded as strong evidence in support of the assumption that these bacteria may be etiologically related to human rheumatic fever.

In general, then, it seems quite clear that there is a very close, probably etiological, relation between *Str. pyogenes* and rheumatic fever, but the pathogenesis of the disease is far from clear.

ACUTE GLOMERULONEPHRITIS

An association of acute hemorrhagic glomerulonephritis with streptococcal infection has been known for many years, beginning, in fact, with Bright's description of the condition in association with scarlet fever as early as 1836. A causal relation between streptococcal infection and this disease appears to be definitely indicated by application of typing methods to give a high correlation between this kind of nephritis and certain types of streptococci.[81, 103, 122, 126]

In contrast to rheumatic fever, which occurs as a complication of *Str. pyogenes* infection with all serological types at a more or less constant rate of around 3 per cent, the attack rate in glomerulonephritis associated with streptococcal infection is highly variable, and extremes of 0.03 and 18 per cent have been observed. The disease may occur in semi-epidemic form, especially in school children. This may be taken to suggest that, unlike rheumatic fever, glomerulonephritis may reflect the variable distribution in time and space of nephritogenic strains of *Str. pyogenes.*[78] In apparent confirmation of this, it has been found that the majority of cases in this country which have been studied bacteriologically have been associated predominantly with *Str. pyogenes* type 12, a lesser number with type 4, and occasionally other serological types may be implicated. An apparently new type of *Str. pyogenes,* the Red Lake strain, occurring in an epidemic of nephritis on an Indian reservation has been described also.[114] A similar association with type 12 has been observed also in Canada and

in England. It is by no means certain that all type 12 strains are nephritogenic, and strains apparently differ among themselves in this respect. Other than this association with serotype, nephritogenic strains of *Str. pyogenes* are not differentiable from those which are not associated with kidney disease.

Glomerulonephritis differs from rheumatic fever also in that it does not tend to recur in the recovered individual, and it is supposed that an effective degree of immunity to the type-specific M antigen persists and that a hypersensitivity is not involved in the disease. Recovery usually seems to be complete, and there seems to be no evidence that chronic nephritis is causally related to prior streptococcal infection.

The disease has been reproduced in rabbits[82, 83] and monkeys[84] by infection with nephritogenic type 12 strains of *Str. pyogenes* or with products of the bacteria administered repeatedly, and in mice with soluble streptococcal products in *in vivo* diffusion chambers.[47, 110] Renal lesions have also been produced in experimental animals by inoculation of soluble antigen-antibody complexes[63, 116] and in parabiosed animals.[54] The evidence shows that kidney pathology results from the antigen-antibody reactions following immunization to streptococcal antigen, and are an indirect, rather than a direct, consequence of the primary infection.

SUBACUTE BACTERIAL ENDOCARDITIS

Infection of the endocardium with the formation of ulcerative lesions may occur with many bacteria. By far the most common are the α-hemolytic streptococci, but staphylococci, β-hemolytic streptococci, and, to a much lesser extent, pneumococci, gonococci, meningococci, Hemophilus, Brucella, Salmonella, etc., are found. With the more highly virulent bacteria the infection is an acute one, but the disease known as subacute bacterial endocarditis, which is no less fatal, is caused almost exclusively by a α-hemolytic streptococci, though in a very few instances anhemolytic streptococci have been implicated.

The infection of the heart valves may be primary or secondary to a focus of infection elsewhere in the body, and an almost indispensable predisposing factor is congenital abnormality or prior damage, as by rheumatic infection, of the heart valves. The source of infection is often the tonsils and periapical infections of the teeth or low-grade infection in the gums. There seems to be an occasional leakage of streptococci from these sources into the blood stream, and any disturbance of the infected areas, such as the extraction of teeth, tonsillectomy, and manipulation of an infected cervix, results in transient bacteremia. The bacteria are rapidly removed from the blood stream by phagocytic cells in the liver, spleen, bone marrow, and elsewhere in the body, but infection of the heart valves may occur when there is an abnormality. Such abnormality may, for example, be a consequence of cardiac damage occurring during an attack of rheumatic fever.

The infection of the endocardium is probably direct rather than from embolism in the smaller vessels at the attachment of the endocardial and subendocardial tissues. According to Grant, Wood, and Jones[30] the bacteria establish in small platelet thrombi on the valvular surfaces, but McNeal, Spence, and Slavkin[62] found that, in experimental endocarditis in the rabbit, the circulating bacteria are phagocytosed by the endothelial cells on the heart valves as well as elsewhere, but are not killed and produce local damage which is covered by a deposit of fibrin; the fibrin protects the streptococci to allow proliferation and the development of a local infection. In any case, during the course of the disease streptococci are shed into the blood stream and may be demonstrated by blood culture. It is significant, especially from the point of view of chemotherapy, that the lesion is not a superficially

situated thrombus, but is, for all practical purposes, an abscess in the substance of the valve.

Species of green streptococci of both the viridans and enterococcus groups are responsible for this disease. In one study it was found that practically all of nearly 200 cultures of green streptococci from clinical sources, including subacute bacterial endocarditis, were either *Str. salivarius* or *Str. fecalis;* and in another[125] the most common species found was *Str. mitis, Str. salivarius* next, and *Str. fecalis* was found least often. The disease process is, however, essentially the same regardless of the species of α-hemolytic streptococcus causing it.

PUERPERAL FEVER

Puerperal fever is a vague term in that some degree of pyrexia is not uncommon immediately following childbirth. A definite febrile response is probably in most instances associated with bacterial infection, but in mild cases the microorganisms are relatively avirulent. The severe infections are due almost entirely to streptococci, the majority *Str. pyogenes.* Infection with other β-hemolytic streptococci occurs, but when these are of groups other than Group A the infections are usually not severe. The anaerobic streptococci are second only to *Str. pyogenes* in importance, being the cause of possibly 20 to 25 per cent of cases of severe puerperal fever; the disease caused by these forms is not as rapidly fulminating as that caused by *Str. pyogenes,* but the case fatality rate is high, possibly 40 per cent. In fatal cases of puerperal fever the infection generalizes to fatal septicemia, and it is probable that the pronounced invasive qualities of the streptococci are responsible for their virulence under these circumstances.

A point that has been of primary interest is the source of the streptococci producing puerperal fever. It seems well established that *Str. pyogenes* occurs only rarely in the female genital tract and is rarely found before labor or during an afebrile puerperium. Lancefield and Hare,[51] for example, reported a series of 855 cultures of the vagina of parturient women; one strain of *Str. pyogenes* was found among sixty-five strains isolated during an afebrile puerperium, and none among thirteen strains isolated before labor. This and similar studies indicate that the source of infection with *Str. pyogenes* is exogenous rather than endogenous. The development of streptococcus typing and a reasonably precise definition of the immunological types has allowed the demonstration of the probable source of the infecting organisms. In one study, slightly over 50 per cent of the streptococci from the patient were identical with those from the nose and throat of attendants, and a little less than 25 per cent identical with those from the nose and throat of the patient. Colebrook[11] reported similar results, the percentages being 58 and 38 respectively. Data such as these show that the source of infection is exogenous with respect to the genital tract and most probably the nose and throat of attending persons or the patient herself in 75 per cent or more of cases. The infection may be air- or dust-borne, but it seems probable that the hands play a significant part in the transfer of infection.

The anaerobic streptococci, on the other hand, are normal inhabitants of the human vagina.[108] In the absence of precise immunological methods for their identification, it may be tentatively concluded that infection with these organisms to produce puerperal fever is endogenous for the most part.

REFERENCES

1. Ablondi, F. B., and Hagan, J. J.: Studies on specificity of streptokinase. Proc. Soc. Exptl. Biol. Med., 1958, **99:**769–772.
2. Barkulis, S. S.: Biochemical properties of a virulent and an avirulent strain of group A hemolytic streptococcus. Ann. N. Y. Acad. Sci. 1960, **88:**1034–1053.
3. Barkulis, S. S., and Jones, M. F.: Studies of streptococcal cell walls. I. Isolation, chemical composition, and preparation of M protein. J. Bact., 1957, **74:**207–216.

4. Barkulis, S. S., Walsh, G., and Ekstedt, R. D.: Studies of streptococcal cell walls. II. Type-specific antigenicity in rabbits. J. Bact., 1958, **76:**109–115.
5. Bartels, E. D., and Riskaer, N.: The bacteriology of erysipelas in clinical light. Acta Med. Scand., 1944, **118:**489–505.
6. Bell, J. F., Owen, C. R., and Jellison, W. L.: Group A streptococcus infections in wild rodents. J. Infect. Dis., 1958, **103:**196–203.
7. Bernheimer, A. W.: Properties of certain rapidly acting bacterial toxins as illustrated by streptolysins O and S. Bact. Rev., 1948, **12:** 195–202.
8. Carlson, A. S., Kellner, A., and Bernheimer, A. W.: Selective inhibition by preparations of streptococcal filtrates of the oxidative metabolism of mitochondria procured from rabbit myocardium. J. Exptl. Med., 1956, **104:**577–587.
9. Catanzaro, F. J., et al.: Effect of oxytetracycline therapy of streptococcal sore throat on the incidence of acute rheumatic fever. Ann. Intern. Med., 1955, **42:**345–357.
10. Coburn, A. F.: The rheumatic fever problem. Am. J. Dis. Children, 1945, **70:**339–347; 348–358.
11. Colebrook, D. C.: The source of infection in puerperal fever due to haemolytic streptococci. Medical Research Council (Great Britain), Spec. Rept. Ser. No. 205, 1935.
12. Commission on Acute Respiratory Diseases: The role of Lancefield groups of beta-hemolytic streptococci in respiratory infections. New England Med. J., 1947, **236:**157–166.
13. Cremer, N., and Watson, D. W.: Rash induction by intracellular products of group A streptococci. Proc. Soc. Exptl. Biol. Med., 1960, **104:**570–573.
14. Cremer, N., and Watson, D. W.: Host-parasite factors in group A streptococcal infections. A comparative study of streptococcal pyrogenic toxins and gram-negative bacterial endotoxin. J. Exptl. Med., 1960, **112:**1037–1053.
15. Cromartie, W. J., Schwab, J. H., and Craddock, J. G.: The effect of a toxic cellular component of group A streptococci on connective tissue. Amer. J. Path., 1960, **37:**79–99.
16. Crowley, N.: Hyaluronidase production by haemolytic streptococci of human origin. J. Path. Bact., 1944, **56:**27–35.
17. Custod, J. T., Lytle, R. I., Johnson, B. H., and Frank, P. F.: Interdependence of hyaluronic acid and M protein in streptococcal aerosol infections in mice. Proc. Soc. Exptl. Biol. Med., 1960, **103:**751–753.
18. Eaton, M. D.: Recent chemical investigations of bacterial toxins. Bact. Rev., 1938, **2:**3–46.
19. Elliott, S. D.: Type and group polysaccharides of group D streptococci. J. Exptl. Med., 1960, **111:**621–630.
20. Eriksson, J.: Streptococcal pneumonia. Four cases presenting specific clinical and bacteriological pictures due to α-streptococci. Acta Med. Scand., 1946, **124:**126–133.
21. Evans, A. C.: Studies on hemolytic streptococci. VIII. *Streptococcus equisimilis.* J. Bact., 1944, **48:**267–284.
22. Evans, A. C., and Chinn, A. L.: The enterococci: With special reference to their association with human disease. J. Bact., 1947, **54:** 495–512.
23. Feinstein, A. R., and Spagnuolo, M.: Mimetic features of rheumatic-fever recurrences. New England J. Med., 1960, **262:**533–540.
24. Finland, M., Samper, B. A., and Barnes, M. W.: Cold hemagglutinins. VI. Agglutinins for an indifferent streptococcus in primary atypical pneumonia and in other conditions and their relation to cold isohemagglutinin. J. Clin. Invest. 1945, **24:**497–502.
25. Florman, A. L., and Weiss, A. B.: Serologic reactions in primary atypical pneumonia. J. Lab. Clin. Med., 1945, **30:**902–910.
26. Foley, G. E., Aycock, W. L., and Cox, R. D.: Serologic types of hemolytic streptococci in scarlet fever in Massachusetts. New England J. Med., 1945, **233:**761–765.
27. Freimer, E. H., Krause, R. M., and McCarty, M.: Studies of L forms and protoplasts of group A streptococci. I. Isolation, growth and bacteriologic characteristics. J. Exptl. Med., 1959, **110:**853–874.
28. Ginsburg, I., and Grossowicz, N.: Group A hemolytic streptococci. I. A chemically defined medium for growth from small inocula. Proc. Soc. Exptl. Biol. Med., 1957, **96:**108–112.
29. Glaser, R. J., and Wood, W. B., Jr.: Pathogenesis of streptococcal pneumonia in the rat. Arch. Path., 1951, **52:**244–252.
30. Grant, R. T., Wood, J. E., Jr., and Jones, T. D.: Heart valve irregularities in relation to subacute bacterial endocarditis. Heart, 1928, **14:** 247–261.
31. Griffith, G. C.: The epidemiology of rheumatic fever, its public health aspects. Amer. J. Pub. Health, 1948, **38:**682–688.
32. Hamburger, M., Jr., et al.: Ability of different types of hemolytic streptococci to produce scarlet fever. J. Amer. Med. Assn., 1944, **124:**564–566.
33. Hamburger, M., Jr., Green, M. J., and Hamburger, V. G.: The problem of the "dangerous carrier" of hemolytic streptococci. II. Spread of infection by individuals with strongly positive nose cultures who expelled large numbers of hemolytic streptococci. J. Infect. Dis., 1945, **77:**96–108.
34. Hamburger, M., Jr., Lemon, H. M., and Platzer, R. F.: The significance of nose to throat carrier ratios in the epidemiology of hemolytic streptococcal infection. Amer. J. Hyg., 1949, **49:**140–147.
35. Hartman, T. L., and Weinstein, L.: Drug sensitivity of hemolytic streptococci isolated from cases of scarlet fever treated with penicillin. Proc. Soc. Exptl. Biol. Med., 1948, **69:**314–316.
36. Hartman, T. L., and Weinstein, L.: The problem of sulfonamide-resistant hemolytic streptococci. New England J. Med., 1948, **238:** 560–563.
37. Hook, E. W., Wagner, R. R., and Lancefield, R. C.: An epizootic in Swiss mice caused by a group A streptococcus, newly designated type 50. Amer. J. Hyg., 1960, **72:**111–119.
38. Hoyne, A. L., and Herzon, H.: *Streptococcus viridans* meningitis: A review of the literature and report of nine recoveries. Ann. Intern. Med., 1950, **33:**879–902.
39. Hugh, R.: Motile streptococci isolated from the oropharyngeal region. Canadian J. Microbiol., 1959, **5:**351–354.
40. Iversen, K.: Streptococcal pneumonia. Acta Med. Scand., 1948, **131:**200–208.

41. Jennings, R. K.: Antigens in scarlatinal erythrogenic toxin demonstrable by the Oudin technic. J. Immunol., 1953, **70:**181–186:
42. Johnson, B. H., and Furrer, W.: Hyaluronic acid: A required component of beta hemolytic streptococci for infecting mice by aerosol. J. Infect. Dis., 1958, **103:**135–141.
43. Johnson, E. E., Stollerman, G. H., Grossman, B. J., and McCullough, H.: Streptococcal infections in adolescents and adults after prolonged freedom from rheumatic fever. I. Results of the first three years of the study. New England J. Med., 1960, **263:**105–111.
44. Kaplan, M.: The concept of autoantibodies in rheumatic fever and in the postcommissurotomy state. Ann. N. Y. Acad. Sci., 1960, **86:**974–991.
45. Keith, J. D.: Modern trends in acute rheumatic fever. Canadian Med. Assn. J., 1960, **83:** 789–796.
46. Kellner, A., Bernheimer, A. W., Carlson, A. S., and Freeman, E. B.: Loss of myocardial contractility in isolated mammalian hearts by streptolysin O. J. Exptl. Med., 1956, **104:**361–373.
47. Kelly, D. K., and Winn, J. F.: Renal lesions produced by group A, type 12, streptococci. Science, 1958, **127:**1337.
48. Kerr, W. J.: Pathogenesis of rheumatic fever. Ann. Intern. Med., 1948, **29:**587–594.
49. Lancefield, R. C.: Differentiation of group A streptococci with a common R antigen into three serological types, with special reference to the bactericidal test. J. Exptl. Med., 1957, **106:**525–544.
50. Lancefield, R. C.: Occurrence of R antigen specific for group A type 3 streptococci. J. Exptl. Med., 1958, **108:**329–341.
51. Lancefield, R. C., and Hare, D.: The serological differentiation of pathogenic and nonpathogenic strains of hemolytic streptococci from parturient women. J. Exptl. Med., 1935, **61:**335–349.
52. Lancefield, R. C., and Perlmann, G. E.: Preparation and properties of type-specific M antigen isolated from a group A, type 1 hemolytic streptococcus. J. Exptl. Med., 1952, **96:**71–82.
53. Lancefield, R. C., and Perlmann, G. E.: Preparation and properties of a protein (R antigen) occurring in streptococci of group A, type 28 and in certain streptococci of other serological groups. J. Exptl. Med., 1952, **96:** 83–97.
54. Lange, K., Wachstein, M., and McPherson, S. E.: Immunologic mechanisms of transmission of experimental glomerulonephritis in parabiosed rats. Proc. Soc. Exptl. Biol. Med., 1961, **106:**13–16.
55. Loosli, C. G., Lemon, H. M., Wise, H., and Robertson, O. H.: Studies on the transmission and control of respiratory disease within army barracks. I. Hemolytic streptococcal contamination of the environment. J. Infect. Dis., 1948, **82:**59–71.
56. MacLennan, J. D.: Streptococcal infection of muscle. Lancet, 1943, **ii:**582–584.
57. Matthew, H., and Gilchrist, A. R.: Subacute bacterial endocarditis and its treatment with penicillin. Edinburgh Med. J., 1949, **56:**25–47.
58. Maxted, W. R.: The indirect bactericidal test as a means of identifying antibody to the M antigen of *Streptococcus pyogenes*. Brit. J. Exptl. Path., 1956, **37:**415–422.
59. McCarty, M. (ed): Streptococcal Infections. Columbia University Press, 1954.
60. McCarty, M.: Variation in group-specific carbohydrate of group A streptococci. II. Studies on the chemical basis for serological specificity of the carbohydrates. J. Exptl. Med., 1956, **104:**629–643.
61. McClean, D.: The capsulation of streptococci and its relation to diffusion factor (hyaluronidase). J. Path. Bact., 1941, **53:**13–27.
62. McNeal, W. J., Spence, M. J., and Slavkin, A. E.: Progressive experimental endocarditis lenta. Amer. J. Path., 1944, **20:**95–105.
63. Miller, F., Benacerraf, B., McCluskey, R. T., and Potter, J. L.: Production of acute glomerulonephritis in mice with soluble antigen-antibody complexes prepared from homologous antibody. Proc. Soc. Exptl. Biol. Med., 1960, **104:**706–709.
64. Mirick, G. S., Thomas, L., Curnen, F. C., and Horsfall, F. L., Jr.: Studies on a nonhemolytic streptococcus isolated from the respiratory tract of human beings. I. Biological characteristics of streptococcus MG. J. Exptl. Med., 1944, **80:**391–406.
65. Mirick, G. S., Thomas, L., Curnen, F. C., and Horsfall, F. L., Jr.: Studies on a nonhemolytic streptococcus isolated from the respiratory tract of human beings. II. Immunological characteristics of streptococcus MG. J. Exptl. Med., 1944, **80:**407–430.
66. Morris, A. J., Chamovitz, R., Catanzaro, F. J., and Rammelkamp, C. H., Jr.: Prevention of rheumatic fever by treatment of previous streptococcal infections. Effect of sulfadiazine. J. Amer. Med. Assn., 1956, **160:**114–116.
67. Morse, S. I., Darnell, J. E., Jr., Thomas, W. A., and Glaser, R. J.: Cardiac lesions in rabbits after pharyngeal infections with group A streptococci. Proc. Soc. Exptl. Biol. Med., 1955, **89:**613–616.
68. Morton, H. E., and Sommer, H. E.: The variation of group C hemolytic streptococci. J. Bact., 1944, **47:**123–128.
69. Niven, C. F., Jr., and Sherman, J. M.: Nutrition of enterococci. J. Bact., 1944, **47:**335–342.
70. Osler, A. G., Buchbinder, L., and Steffen, G. I.: Experimental enterococcal food poisoning in man. Proc. Soc. Exptl. Biol. Med., 1948, **67:**446–449.
71. Paul, H.: Deaths from scarlet fever in the twentieth century. Canadian J. Pub. Health, 1955, **46:**363–367.
72. Perry, W. D., et al.: Transmission of group A streptococci. I. The role of contaminated bedding. Amer. J. Hyg., 1957, **66:**85–95.
73. Perry, W. D., Siegel, A. C., and Rammelkamp, C. H., Jr.: Transmission of group A streptococci. III. The role of contaminated dust. Amer. J. Hyg., 1957, **66:**96–101.
74. Pfeiffer, H.: Ueber Enterokokkenpneumonie (Ein Beitrag zur Frage der "Atypischen Pneumonie"). Med. Klin., 1950, **45:**104–107.
75. Public Health Laboratory Service: Serotypes of *Streptococcus pyogenes:* Their relative prevalence in England and Wales, 1952–1956. Monthly Bull. Ministry of Health and Pub. Health Lab. Service, 1957, **16:**163–172.
76. Pulvertaft, R. J. V.: Bacteriology of war wounds. Lancet, 1943, **ii:**1–2.
77. Quinn, R. W., Denny, F. W., and Riley, H. D.: Natural occurrence of hemolytic streptococci

in normal school children. Amer. J. Pub. Health, 1957, **47:**995–1008.
78. Rammelkamp, C. H., Jr., and Weaver, R. S.: Acute glomerulonephritis. The significance of variations in the incidence of the disease. J. Clin. Invest., 1953, **32:**345–358.
79. Rantz, L. A., and Boisvert, P. J.: Streptococcal fibrinolysin (streptokinase). A study of this substance and its antibody in group A hemolytic streptococcus sore throat. Amer. J. Med., 1948, **5:**24–32.
80. Rao, S., and Moloney, P. J.: Estimation of potency of scarlatinal antitoxin by a combined flocculation and rabbit skin test method. J. Immunol., 1950, **64:**57–64.
81. Reed, R. W.: An epidemic of acute nephritis. Canadian Med. Assn. J., 1953, **68:**448–455.
82. Reed, R. W., and Matheson, B. H.: Experimental nephritis due to type specific streptococci. I. The effect of single exposure to type 12 streptococci. J. Infect. Dis., 1954, **95:**191–201.
83. Reed, R. W., and Matheson, B. H.: Experimental nephritis due to type specific streptococci. II. The effect of repeated exposure to type 12 streptococci. J. Infect. Dis., 1954, **95:**202–212.
84. Reed, R. W., and Matheson, B. H.: Experimental nephritis due to type specific streptococci. IV. The effect of type 12 nephrotoxin in monkeys. J. Infect. Dis., 1960, **106:**245–249.
85. Report: Prevention of rheumatic fever. Second report of the Expert Committee on Rheumatic Diseases. World Health Organization, Palais des Nations, Geneva, Technical Rept. Ser. No. 126, 1956.
86. Rhoads, P. S.: Present status of immunization to and treatment of scarlet fever. Amer. J. Dis. Children, 1949, **77:**244–252.
87. Roberson, J. S., and Schwab, J. H.: Endotoxic properties associated with cell walls of group A streptococci. J. Infect. Dis., 1961, **108:**25–34.
88. Rosebury, T.: The aerobic non-hemolytic streptococci. A critical review of their characteristics and pathogenicity with special reference to the human mouth and to subacute bacterial endocarditis. Medicine, 1944, **23:**249–280.
89. Rothbard, S., and Watson, R. F.: Variation occurring in group A streptococci during human infection. Progressive loss of M substance correlated with increasing susceptibility to bacteriostasis. J. Exptl. Med., 1948, **87:**521–533.
90. Rowen, R., and Bernheimer, A. W.: The toxic action of preparations containing the oxygen-labile hemolysin of *Streptococcus pyogenes*. V. Mechanism of refractoriness to the lethal effect of the toxin. J. Immunol., 1956, **77:**72–79.
91. Schwab, J. H.: An intracellular hemolysin of group A streptococci. I. Influence of sonic energy and pH on hemolytic activity. J. Bact., 1956, **71:**98–99.
92. Schwab, J. H.: An intracellular hemolysin of group A streptococci. II. Comparative properties of intracellular hemolysin, streptolysin S and streptolysin O. J. Bact., 1956, **71:**100–107.
93. Schwab, J. H., and Cromartie, W. J.: Studies on a toxic cellular component of group A streptococci. J. Bact., 1957, **74:**673–678.
94. Schwab, J. H., Cromartie, W. J., and Roberson, B. S.: Identification of a toxic cellular component of group A streptococci as a complex of group-specific C polysaccharide and a protein. J. Exptl. Med., 1959, **109:**43–54.
95. Schwab, J. H., and Sharpless, E. A.: An intracellular hemolysin of group A streptococci. III. Immunological studies. J. Bact., 1960, **79:**488–495.
96. Seal, J. R.: Oral penicillin prophylaxis of streptococcal infections. Amer. J. Pub. Health. 1955, **45:**662–672.
97. Seal, J. R., Mogabgab, W. J., Friou, G. J., and Banta, J. E.: Penicillin prophylaxis of epidemic streptococcal infections. I. The epidemic and the effects of prophylaxis on the clinical manifestations of acute streptococcal and nonstreptococcal respiratory infections. J. Lab. Clin. Med., 1954, **44:**727–753.
98. Seal, J. R., Mogabgab, W. J., Friou, G. J., and Banta, J. E.: Penicillin prophylaxis of epidemic streptococcal infections. II. The effects of small and large doses of oral penicillin on epidemic streptococcal infections and on carriers of group A streptococci. J. Lab. Clin. Med., 1954, **44:**831–859.
99. Seelemann, K., and Neuman, H.: Moderne Scharlachbehandlung und Antistreptolysinreaktion. Deut. med. Woch., 1954, **79:**1825–1828, 1837.
100. Sharpless, E. A., and Schwab, J. H.: An intracellular hemolysin of group A streptococci. IV. Lethal activity in mice. J. Bact., 1960, **79:**496–501.
101. Sherman, J. M.: The streptococci. Bact. Rev., 1937, **1:**1–97.
102. Sherman, J. M.: The enterococci and related streptococci. J. Bact., 1938, **35:**81–93.
103. Siegel, A. C., Rammelkamp, C. H., Jr., and Griffeath, H. I.: Epidemic nephritis in a school population. The relation of hematuria to group A streptococci. Pediatrics, 1955, **15:**33–44.
104. Smyth, C. J., et al.: Rheumatism and arthritis: Review of American and English literature of recent years. Ann. Intern. Med., 1960, **53:**1–365.
105. Soloman, S., and Kalkstein, M.: Pneumonia due to the *Streptococcus viridans*. Amer. J. Med. Sci., 1943, **205:**765–770.
106. Spaun, J., Bentzon, M. W., Olesen Larson, S., and Hewitt, L. F.: International standard for antistreptolysin O. Bull. W. H. O., 1961, **24:**271–279.
107. Stollermann, G. H., Rusoff, J. H., and Hirschfeld, I.: Prophylaxis against group A streptococci in rheumatic fever. The use of single monthly injections of benzathine penicillin G. New England J. Med., 1955, **252:**787–792.
108. Stone, M. L.: Bacteriologic characteristics of anaerobic streptococci recovered from postpartum patients. Amer. J. Obstet. Gyn., 1951, **42:**68–74.
109. Symposium: Panel discussion: rheumatic fever: summary of present concepts. Pediatrics, 1949, **3:**680–710.
110. Tan, E. M., Hackel, D. B., and Kaplan, M. H.: Renal tubular lesions in mice produced by group A streptococci grown in intraperitoneal diffusion chambers. J. Infect. Dis., 1961, **108:**107–112.
111. Thomas, H. M., Jr.: The role of alpha hemolytic streptococcus in pneumonia. Bull. Johns Hopkins Hosp., 1943, **72:**228–231.

112. Thomas, L. J., Mirick, G. S., Ziegler, J. E., Jr., and Horsfall, F. L., Jr.: Studies on primary atypical pneumonia. II. Observations concerning the relationship of a non-hemolytic streptococcus to the disease. J. Clin. Invest., 1945, **24:**227–240.
113. Tillett, W. S.: The fibrinolytic activity of hemolytic streptococci. Bact. Rev., 1938, **2:**161–216.
114. Updyke, E. L., Moore, M. S., and Conroy, E.: Provisional new type of group A streptococci associated with nephritis. Science, 1955, **121:** 171–172.
115. Wagner, B. M.: Studies in rheumatic fever. III. Histochemical reactivity of the Aschoff body. Ann. N. Y. Acad. Sci., 1960, **86:**992–1008.
116. Wakim, K. G., and McKenzie, B. F.: The influence of streptococcal toxin on the course of canine experimental nephrosis. J. Lab. Clin. Med., 1957, **50:**410–416.
117. Wannamaker, L. W.: The differentiation of three distinct deoxyribonucleases of group A streptococci. J. Exptl. Med., 1958, **107:**797–812.
118. Watson, D. W.: Host-parasite factors in group A streptococcal infections. Pyrogenic and other effects of immunologic distinct exotoxins related to scarlet fever toxins. J. Exptl. Med., 1960, **111:**255–284.
119. Watson, D. W., Rothbard, S., and Swift, H. F.: Type-specific protection and immunity following intranasal inoculation of monkey with group A hemolytic streptococci. J. Exptl. Med., 1946, **84:**127–142.
120. Weinstein, L.: Antigenic dissimilarity of streptokinases. Proc. Soc. Exptl. Biol. Med., 1953, **83:**689–691.
121. Weinstein, L., Boyer, N. H., and Goldfield, M.: Rheumatic heart disease in scarlet-fever patients treated with penicillin. A follow-up study after seven years. New England J. Med., 1955, **253:**1–7.
122. Wertheim, A. R., et al.: The association of type specific hemolytic streptococci with acute glomerulonephritis at the Presbyterian and Babies Hospitals, New York, N. Y. in the years 1936–1942. J. Clin. Invest., 1953, **32:** 359–363.
123. Wiley, G. G., and Wilson, A. T.: The occurrence of two M antigens in a certain group A streptococcus related to type 14. J. Exptl. Med., 1961, **113:**451–465.
124. Williams, R. E. O.: Laboratory diagnosis of streptococcal infection. Bull. W. H. O. 1958, **19:**163–176.
125. Willius, F. A.: Cardiac clinics; subacute bacterial endocarditis; bacteriology. Proc. Staff Meetings Mayo Clinic, 1944, **19:**380–384.
126. Wilmers, M. J., Cunliffe, A. C., and Williams, R. E. O.: Type-12 streptococci associated with acute haemorrhagic nephritis. Lancet, 1954, **ii:**17–18.
127. Wilson, A. T.: Nucleic acid derivatives as growth factors for certain group A hemolytic streptococci. Proc. Soc. Exptl. Biol. Med., 1945, **58:**249–254.
128. Wilson, A. T.: The leukotoxic action of streptococci. J. Exptl. Med., 1957, **105:**463–484.
129. Wilson, A. T.: The relative importance of the capsule and the M-antigen in determining colony form of group A streptococci. J. Exptl. Med., 1959, **109:**257–270.
130. Wilson, M. G.: Hereditary susceptibility in rheumatic fever. Eugenics Quart., 1956, **3:**38–44.
131. World Health Organization: The trend of scarlet fever in recent years. Epidemiol. Vital Stat. Rept. W. H. O., 1951, **4:**354–366.
132. World Health Organization. Scarlet fever. Its recent trend. Epidemiol. Vital Stat. Rept. W. H. O., 1952, **5:**624–629.

Chapter 18

THE PNEUMOCOCCI[47]

THE BACTERIUM most commonly found in pneumonia in man is a small lanceolate micrococcus which has been variously termed *Micrococcus pneumoniae, M. lanceolatus, Streptococcus pneumoniae* or, more briefiy, the *pneumococcus* or *Fränkel's pneumococcus. Diplococcus pneumoniae* is now the commonly accepted formal name.

Of the generally recognized anatomical types of pneumonia, lobar or acute croupous pneumonia, bronchopneumonia or lobular pneumonia, and capillary bronchitis or bronchiolitis, lobar pneumonia is nearly always due to the pneumococcus, though other bacteria are occasionally involved. Perhaps the best quantitative data are those assembled by Rumreich and his co-workers[40] in a 2-year study in six states representing high and low pneumonia rates. These are summarized in the accompanying table. It is evident that the pneumococcus is by far the commonest cause of pneumonia. The microorganisms associated with bronchopneumonia are varied, and their source is probably almost always the nasopharynx.[42, 43]

The pneumococcus was discovered independently in France in 1881 by Pasteur, who inoculated rabbits with the saliva of a child dead of rabies, and by Sternberg in the United States, but was not known to be associated with disease in man before the extensive investigations of Fränkel and Weichselbaum, who demonstrated conclusively the etiological relation of this bacterium to pneumonia in man.

Morphology and Staining. The pneumococcus is typically a small, slightly elongated coccus, one end of which is

The Incidence of Etiological Agents in Pneumonia*

CAUSATIVE ORGANISM	LOBAR PNEUMONIA	BRONCHO-PNEUMONIA	UNSPECIFIED	ALL PNEUMONIAS
Pneumococcus	82.48	65.79	77.48	77.71
Hemolytic streptococcus	2.00	3.33	3.99	2.65
Other streptococci	1.30	2.99	1.33	1.70
Staphylococcus	.82	2.00	1.38	1.19
Friedländer's bacillus	.15	.13	.28	.17
Influenza bacillus	.06	.25	.11	.15
Tubercle bacillus	.....	.08	.....	.02
Fungi	.....	.02	.....	.....
Virus	.....	.....	.07	.01
No significant organism recorded	13.19	25.41	15.38	16.44
Number of cases	15,420	6,092	4,290	25,802

* In six representative states over a 2-year period as compiled by Rumreich et al.[40]

Figure 87. Pneumococcus, pure culture. Note the typical lanceolate shape and diplococcus arrangement. Fuchsin; × 1050.

pointed or lance-shaped. The cocci commonly occur in pairs (diplococci), but variations both in grouping and in size and form of individual cells are frequently observed. Chain formation is common, especially in artificial mediums, although the chains are usually shorter than those of *Str. pyogenes*. Oval and elongated bacillary forms sometimes occur. The pneumococcus is nonmotile and does not form spores. A well-defined capsule envelops the pneumococci in animal exudates but, except in certain strains or in certain mediums, is less readily demonstrable in cultures grown outside the body. Capsules may often be found in milk cultures and in mediums containing blood or serum.

The pneumococcus is readily stained with the aniline dyes and is generally gram-positive, although there is a tendency to become gram-negative in older cultures, and occasional strains are found to be gram-negative. In stained preparations the capsule may often be seen as an unstained halo surrounding the cells; it may be stained by special methods.

The colonies of pneumococcus on infusion agar or blood agar are typically small, moist, translucent, and granular, with well-defined edges. These bacteria are α-hemolytic on blood agar, and the colonies appear surrounded by a zone of greenish discoloration on this medium and resemble the colonies of green-producing streptococci, so closely as to be practically indistinguishable from them.

Physiology. Some pneumococci grow upon the ordinary nutrient (beef extract) culture mediums but many do not, and, in any case, growth is sparse. Nutritional requirements are complex. A number of semisynthetic mediums have been developed, based on gelatin or casein hydrolysate, and supplemented with vitamins and various other growth accessory substances, and amino acids.[1, 9, 10, 19, 37, 38] The requirement for added choline, nicotinic acid, and pantothenic acid appear to be quite general among the serotypes, and biotin and ascorbic acid are required by some strains. Growth is often markedly stimulated by the inclusion of purines and pyrimidines, but these do not appear to be essential. Amino acid requirements are complex and varied from strain to strain. Of the common serotypes, type 3 appears to be more exacting, but strain differences may be as great, or greater, than differences between serotypes.

Growth on infusion mediums, particularly those enriched by the addition of whole blood, takes place at 37° C. Litmus milk is promptly acidified and often, but not invariably, coagulated. The temperature range over which these bacteria may be grown is relatively narrow (25° to 42° C.), and they are sensitive to variations in pH from the optimum of 7.8, the limiting acidity and alkalinity being 6.5 and 8.3 respectively. The pneumococcus is a facultative anaerobe, although certain other species of Diplococcus are obligate anaerobes.

In general, sugars are actively fer-

mented with the production of large amounts of lactic acid and small amounts of volatile acid and ethyl alcohol. Differential fermentations are of no particular value in the classification of these microorganisms except in the case of inulin, which serves to differentiate the pneumococci and the green streptococci.

The pneumococci appear to be structurally delicate organisms and autolyze much more readily than most other kinds of bacteria. Evidences of protein hydrolysis accompany autolysis, and it appears that lysis is a consequence of the activity of intracellular ferments.[36] Perhaps associated with the autolytic process is the lysis of pneumococci by bile and bile salts. The so-called bile solubility of pneumococci is practically a constant characteristic, although different strains vary in their sensitivity to bile as they do in the tendency to autolysis. Rabbit or ox bile may be used and added to a young broth culture in the proportion of 10 to 20 per cent. Solutions of pure bile salt (sodium taurocholate) are preferable to ox bile because they may be sterilized and the concentration (10 per cent) controlled. Heat-killed pneumococci are not bile-soluble. Sodium lauryl sulfate and similar detergents will also lyse pneumococci.[11, 20]

Pneumococci may, therefore, be distinguished from streptococci by their bile-solubility and, less definitely, their ability to ferment inulin, in addition to their greater pathogenicity for mice and the characteristics of the colonies on agar.

Peroxide is found in considerable quantities in pneumococcus cultures after prolonged incubation because of the lack of catalase in these microorganisms. This, coupled with their sensitivity to peroxide, results in the autosterilization of cultures kept in the incubator for many days. Cultures in blood broth, however, remain viable for several weeks in the refrigerator, and the bacteria may be preserved for months in the cold in vacuum-desiccated spleens of infected mice.

The pneumococci are more sensitive to the bactericidal activity of the usual antiseptics than are many other bacteria. Soaps, such as ricinoleate and oleate, are pneumococcidal in relatively high dilutions, 0.04 and 0.004 per cent respectively, and other substances such as phenol and mercuric chloride are also highly effective in the destruction of these bacteria. Quinine and some of its derivatives have antipneumococcal activity but do not inhibit α-hemolytic streptococci; optochin-containing discs, similar to antibiotic discs, are available for the differentiation of pneumococcus and green streptococci.

Toxins. The severe intoxication observed in pneumococcus infection in man is suggestive of the formation of some toxin by this bacterium. The existence of such a toxin has never been demonstrated, however, and the pneu-

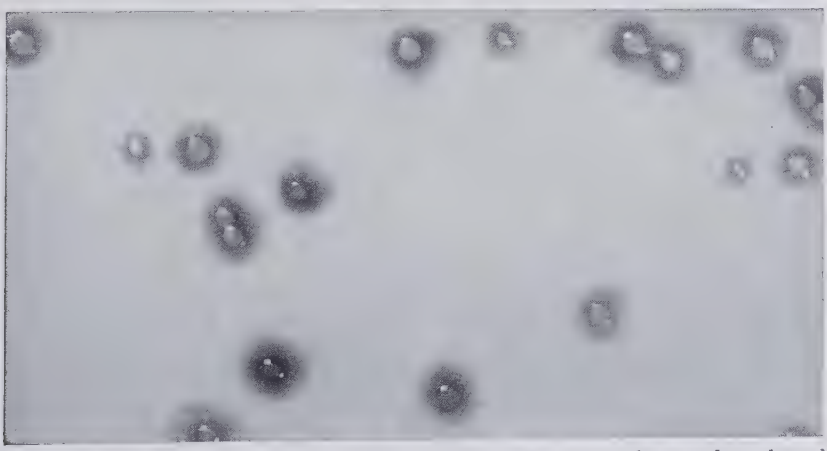


Figure 88. Colonies of the pneumococcus on blood agar. The areas of green hemolysis have been accentuated in the photograph. × 3.

mococcus does not produce a toxin analogous to those of the diphtheria and tetanus bacilli.

Other toxic substances are produced by this microorganism. That there is α-hemolysis on blood plates has already been noted, and there is in addition a filterable hemolysin active on sheep, guinea pig, and human erythrocytes.[34] The concentrated hemolysin is reported to have lethal and dermotoxic properties. The pneumococcus also produces a leucocidin[35] and a necrotizing substance similar to that formed by some of the staphylococci. Many strains produce hyaluronidase, especially when cultivated in mediums containing hyaluronic acid.[26] A purpura-producing substance which is nonantigenic and appears to be a cleavage product of pneumococcal protein has been described by a number of workers. Injected into white mice, extracts of pneumococci produce a purpuric condition manifested as a dark blue discoloration of the skin of the feet, tail, ears, nose, and genitals.[27] It has been observed too that virulent strains are able to utilize extracellular breakdown products of nucleic acids, which stimulate both respiration rate and DNA synthesis.[18]

Although preparations containing these activities have been reported to increase the virulence of relatively avirulent pneumococcus strains when injected simultaneously with the bacteria, the virulence of pneumococci is directly dependent, not on the formation of such toxic substances, but on the production of specific soluble substance and encapsulation.

Classification. The pneumococci are closely related to the streptococci, but the degree of intimacy of the relationship is as yet open to question. Some workers regard these microorganisms as but a species of streptococcus and designate them as *Str. pneumoniae.* In general, however, it is customary to consider the pneumococci as a distinct genus, a practice which is justified by considerations of the sum of the characteristics of the pneumococci and the clinical and epidemiological aspects of the pneumonias. According to Bergey's classification, the tribe Streptococceae is made up of three genera: (1) Diplococcus, of which the type species is *D. pneumoniae* or the pneumococcus; (2) Streptococcus, with *Str. pyogenes* as the type species; and (3) Leuconostoc, a group of gas-forming, chain-producing cocci found in milk, fermenting vegetables, and slimy sugar solutions.

The genus Diplococcus includes six species in all, the five in addition to the pneumococcus being obligate anaerobes. *D. paleopneumoniae* closely resembles the pneumococcus except for its anaerobic character, occurs normally in the buccalpharyngeal cavity, and is reported to be highly pathogenic. *D. plagarumbelli* has been found in septic wounds, and the remainder, *D. magnus, D. con-*

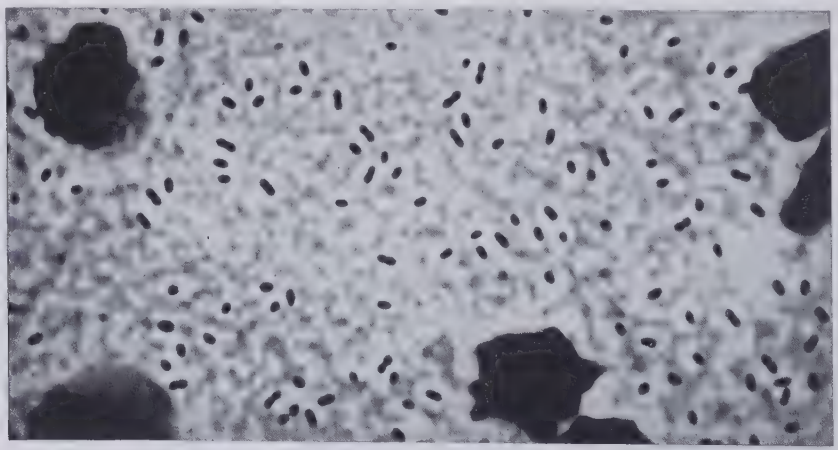


Figure 89. Pneumococcus in the peritoneal fluid of a mouse. Note the capsules. Fuchsin; × 2200.

stellatus, and *D. morbillorum*, appear to be normal inhabitants of the mouth and intestinal tract and have been found in lymphoid tissue such as in the tonsils and appendix.

Pneumococcus types. A point that has been of somewhat more interest than the formal taxonomic position of the pneumococci is the subdivision of these bacteria into types. The pneumococci contain two kinds of antigen. One, the so-called somatic antigen, is a constituent of the cell substance proper and is immunologically identical in all pneumococci. The other, the polysaccharide haptene or specific soluble substance (SSS), is type-specific and serves to differentiate the immunological types of pneumococci from one another. The polysaccharide of type 3 has been the most thoroughly studied chemically and types 1, 2, 5 and 6 to a lesser extent (Chap. 3).[23, 28] These substances have been isolated from most of the other types also. The presence of SSS masks the somatic antigen, and antiserums to encapsulated pneumococci are sharply type-specific. These immunological types are designated by numerals, and the numbering of the types, although purely arbitrary, is a generally accepted convention.

Observations by Dochez and Gillespie in 1913 showed that the pneumococci found in cases of lobar pneumonia can be divided into four distinct groups, designated as types 1, 2, and 3 and Group IV, on the basis of specific agglutination and protection tests. Of these, types 1, 2, and 3, found in the majority of cases of pneumonia and producing the most severe types of infection, have specific immunological character, while Group IV is immunologically heterogeneous and is made up of all pneumococci not belonging to the first three types.

The types making up Group IV were then sorted out by Cooper and her associates in this country, and by Danish workers, to give a great many serological types of pneumococcus. There are two different nomenclatures for the pneumococcal types,[29, 30] the Danish system which is an extension of Cooper's system in which serologically related types are grouped together as subtypes; and the system prepared by Eddy[12] under the auspices of the U.S. Public Health Service and the American Public Health Association, in which the differentiable types are given consecutive numbers irrespective of antigenic relationships. There are eighty differentiable types, but some are antigenically related to one another; there is, for example, partial cross-reaction between types 2 and 5, between 3 and 8, between 7 and 18, and between 15 and 30. Rarely, mixed types, containing antigens characteristic of more than one differentiable type, are found.[16] Mixed types have been produced artificially by transformation reactions[7] (see below), and such types are considered to contain two capsular genomes, one mutated and the other normal, which interact to give the observed phenotypic expression.[8]

Diplococcus mucosus. These immunological types are culturally indistinguishable with the exception of type 3, which stands somewhat apart from the other pneumococci in that it produces a heavy mucoid growth because of its luxuriant capsule formation. It is considered by some to be a separate species, *D. mucosus*. Many cultures show a marked tendency to form chains, and the dividing line between *D. mucosus* and "*Str. mucosus*" is not a sharp one, if indeed any distinction should be made. These coccoid, heavily capsulated bacteria for the most part ferment inulin and are soluble in bile, so that the tendency is to group them with the pneumococci rather than the streptococci. A few mucoid strains have been reported which are bile-insoluble and non-inulin-fermenting.

Pneumococcus typing. The serological typing of pneumococci may be carried out in a number of ways but is not a routine diagnostic procedure. The various methods that have been developed are basically immunological but differ in technical detail. The immunological procedures are of three kinds: (1) the agglutination of the pneumococci with

type-specific antiserum; (2) the precipitation of SSS with type-specific antiserum, and (3) the Quellung reaction. The first two have been discussed in previous sections and need not be considered further here.

The Quellung phenomenon was described by Neufeld in 1902 and since then has come into general use. A suspension of pneumococci is mixed with undiluted antiserum (rabbit serum is preferable to horse serum) on a slide or cover glass, a small amount of Löffler's alkaline methylene blue is added to facilitate observation, and the preparation is examined under the microscope. In the presence of homologous immune serum there is a marked apparent swelling of the capsule without any obvious change in the size of the bacterial cell itself; no such swelling is observed with heterologous serums. The reaction takes place rapidly, and the swelling is usually apparent within a few minutes. This apparent swelling of the capsule in the presence of specific antiserum is discussed elsewhere (Chap. 3).

The use of serum pools considerably facilitates typing, especially the identification of the higher types. The incidence of the various types determines the most advantageous combinations of antiserums. A group of combinations often recommended for use in this country is: (*a*) 1, 2, 7; (*b*) 3, 4, 5, 6, 8; (*c*) 9, 12, 14, 15, 17; (*d*) 10, 11, 13, 20, 22, 24; (*e*) 16, 18, 19, 21, 28; (*f*) 23, 25, 27, 29, 31, 32. Monospecific antiserums are required also. The pneumococcus to be typed is tested with each pool and then with the component antiserums of the pool with which it reacts. Thus a pneumococcus may be identified in twelve or less tests.[13, 14, 15]

Variation. The smooth and rough variants that have been found in a variety of bacteria may also be observed in the pneumococcus. As in other cases, there are various intermediate colonial types between the two extremes, and the pneumococcus is virulent in the smooth form and almost completely avirulent in the rough form. The change from smooth to rough is reflected in the microscopic morphology of the cells as a loss of capsule. According to Austrian,[4, 5] colonial type involves the formation of filaments of pneumococci as well as capsule formation; the typical virulent strain is the nonfilamentous capsulated form. These factors vary independently, as shown by transformation reactions, to give the possible combinations, including a filamentous noncapsulated type which had not been explicitly recognized earlier.

Since type specificity is determined by the SSS, it follows that the change from smooth to rough is accompanied by a complete loss of type specificity; the somatic antigen is predominant and, irrespective of original type, the pneumococci become immunologically identical. The dissociative change may be reversed, although with difficulty, by animal passage or by cultivating the R form in the presence of anti-R immune serum or in the presence of heat-killed cells from a smooth culture.

Transformation of types. The experimental interconversion of pneumococcal types by direct transduction using an R form of pneumococcus and polymerized deoxyribonucleic acid prepared from a heterologous type has been described elsewhere (Chap. 7) together with changes in serotype and other characteristics by phage-mediated transduction. These phenomena have been intensively studied with the pneumococcus, but the extent to which such changes may occur in nature is unknown.

Pathogenicity for Man.[21] As indicated above, lobar pneumonia is the most important pneumococcal infection in man. The bacteria are not confined to the lung for they may migrate from this seat of infection through the nasal passages or be distributed via the vascular system to various parts of the body, to give rise to localized foci of infection. Pneumococcemia is of frequent occurrence; in one series of cases studied[46] over a 14-year period, bacteremia averaged 22.8 per cent, the annual averages ranging from 16.4 to 49 per cent. A number of workers emphasize the prognostic value of blood culture, and in most in-

stances both the case fatality rate and the incidence of purulent complications are considerably higher when pneumococci are present in the blood stream.

Among the pathological processes that occur as complications and sequelae of pneumococcus pneumonia, or, it may be noted, as independent and primary affections, are inflammations of the pleura, pericardium, and meninges. The last continues to be a significant problem for the case fatality rate is extremely high, and it does not respond to chemotherapy as well as many of the other pneumococcal infections.[2, 39, 45] Meningitis and otitis media are frequently secondary to pneumonia, and the connection between inflammation of the middle ear and meningeal infection has often been observed. To those inflammatory conditions of pneumococcal origin might be added a long list of others provoked by the same organism. There appear to be few, if any, organs or tissues that are not under some circumstances subject to attack. Sinusitis, parotitis, conjunctivitis, peritonitis, and a great variety of other affections are occasionally due to pneumococci. In general, pneumococcus infections of this kind tend to a more favorable outcome than similar infections with streptococci or staphylococci.

Resistance to pneumococcal infection is, in large part, a matter of individual predisposition, and the mere presence of pneumococci in the upper respiratory tract is not sufficient to bring about pulmonary infection. Specific immunity plays a negligible part in resistance, but the complex of factors associated with a state of physiological well-being is of the greatest significance. A preliminary depression of resistance by other infections, severe or sudden exposure to cold, fatigue, and other predisposing factors is an almost invariable preliminary to pneumococcus infection. The part played by pneumococcus pneumonia in the fatal termination of many diseases, for example, is well known.

The pathogenicity of pneumococcus types. The case fatality of pneumococcus pneumonia is relatively high and indicative of the pathogenicity of these bacteria once they have become established in the lungs. The pneumococcus types differ from one another in this respect; the case fatality in type 1 infections is 25 to 30 per cent, that of type 2 about 40 per cent, that of type 3, 40 to 60 per cent, and that of Group IV infections perhaps 15 to 20 per cent. Because of the relatively recent differentiation of the types comprising Group IV, data on case-fatality rates for these types are very meager as yet. The frequency of occurrence of the pneumococcal types in lobar pneumonia and bronchopneumonia is given in figure 90. Here the ten leading types are: 1, 2, 3, 4, 5, 6, 7, 8, 14, and 19. In some series, type 2 is more frequent than type 3, and in others, including this one, the reverse is true. In a subsequent study of type incidence in the period 1952–1957, the commonest types were 3, 7 and 1, in that order, and in that series type 2 was rare.[6]

Pneumococcus carriers. As a strict parasite, the pneumococcus is found in man rather than in his environment. Healthy carriers of these bacteria are common; 40 to 60 per cent of groups of persons examined have been found to harbor pneumococci in the upper respiratory tract. This proportion is variable, being relatively high during the cold months of the year and higher among groups of contacts than among noncontacts. The carrier state is not permanent but rather sporadic and intermittent; many persons may carry these bacteria for a short time, particularly while having colds and other infections of the upper respiratory tract, while others may carry them for longer periods. There is also a seasonal fluctuation in that increased numbers are found in the winter months.

The great majority of pneumococci found in carriers are the relatively less virulent types of Group IV. Types 1, 2, and 3 are much less frequently found, but an appreciable portion of the population may harbor these types at any one time (see table). In the study made by Smillie, Calderone, and Onslow[41] almost every type of pneumococcus was encountered; many individuals carried two

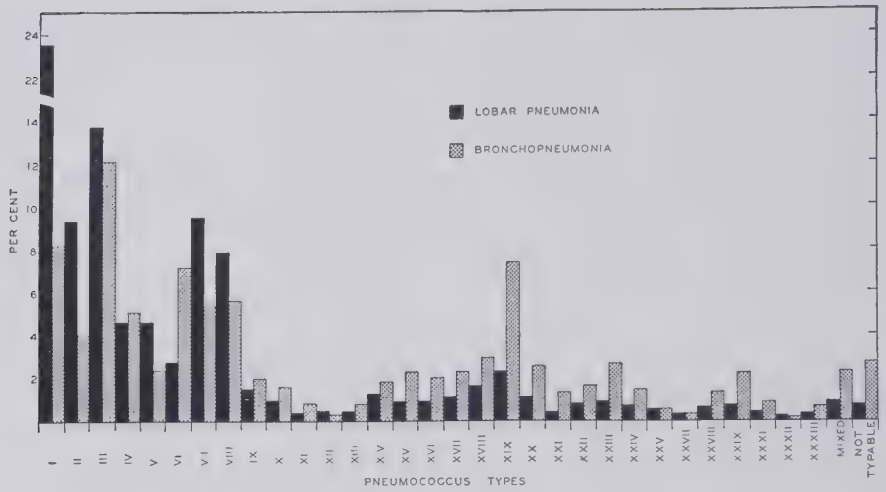


Figure 90. The incidence of pneumococcus types in lobar and bronchopneumonia observed in a 2-year survey in California, Colorado, Illinois, Louisiana, New Jersey, and Missouri in which pneumococci from 12,447 cases of lobar pneumonia and 3847 of bronchopneumonia were typed. The relative predominance of type 3 over type 2 is sometimes observed. Note the more common occurrence of higher types in bronchopneumonia, the relative incidence of mixed types, *i.e.*, those reacting to more than one antiserum, and the incidence of types other than the first thirty-three. Data from Rumreich et al.[40]

or more types simultaneously. The types most commonly found were 3, 7, 21, 25, and 11 in that order of frequency. It is not clear whether an appreciably effective immunity is developed by the carrier.

The Epidemiology of Pneumococcus Pneumonia.[17] Pneumococci are disseminated chiefly through the secretions and discharges of the mouth and upper respiratory tract by direct contact between persons. Droplet infection undoubtedly plays a large part in the transmission of these microorganisms and perhaps accounts for the seasonal incidence of the disease and the increased frequency of carriers during the cold months of the year.

While it is obvious that pneumococcus infection is always exogenous in the last analysis, for practical purposes it is probably endogenous in a large proportion of cases. With a high carrier rate pneumococci are frequently present in the normal individual, and when resistance is reduced to a sufficiently low level they are able to set up an infection. The high incidence of pneumococci of types 1 and 2 as contrasted with the low carrier incidence of these types, together with the occurrence of seeming epidemics of pneumococcus pneumonia on a small scale, has been regarded by some as evidence of exogenous infection. It is more probable, however, that the greater virulence of some pneumococcus types operates as a selective factor to disturb the random distribution of types in pneumococcus pneumonia. It is known, for example, that minor respiratory infections may be common or epidemic in small groups such as a family. If a given type of pneumococcus of high virulence invades and spreads within the group so that a high proportion of the individuals become carriers, the operation of factors which reduce resistance in the group may result in one or more members coming down with pneumonia due to the type carried. Such a sequence of events was observed in a group of children in a nursery.[44] The group was invaded by a virulent type 14 pneumococcus which caused no harm, but when the individual developed an acute respiratory infection, the dormant pneumococcus spread to the middle ear, conjunctivas, and lungs. Such children were sent to the hospital ward and carried the pneumococcus, which spread to most of the

Pneumococcus Carriers*

	PERSONS EXAMINED			INCIDENCE OF CARRIERS				
		Cases Found			Per Cent of Incidence of Types			
	Total	Number	Per Cent	Total†	1	2	3	Group IV
Non-contacts	2332	1000	42.9	1027	0.5	0.9	8.4	34.2
Contacts	1782	977	54.8	1018	3.3	2.7	10.0	41.0

* Modified from Heffron.[21]

† The incidence of types is greater than the incidence of carriers because in some instances more than one type was found.

children there. Again, the infection was activated, sometimes with serious consequences, on the development of respiratory infection. Under appropriate circumstances pneumococcal pneumonia may occur in epidemic form. One such epidemic, occurring among military personnel, which was studied in detail,[24] showed too that the most significant factors were the carrier rate, and the rate of nonbacterial respiratory disease as a predisposing factor.

Other epidemiological characteristics of pneumococcus pneumonia include seasonal incidence, which corresponds roughly with the carrier rate; the age incidence, characterized by high morbidity and mortality in infants and the aged; the higher incidence in males than in females; and the apparent greater susceptibility of the Negro as contrasted with the white race.

Bacteriological Diagnosis of Pneumococcus Infections.[31] The pneumococcus may be isolated by culture or animal inoculation from specimens such as sputum, pleural exudate, blood, spinal fluid, and pus. Blood agar is the medium of choice, the bacteria growing up in 24 hours as small colonies surrounded by a zone of green hemolysis. It is not possible to distinguish them from α-hemolytic streptococci by colonial or microscopic morphology, but differentiation may be made by the fermentation of inulin and bile solubility of the pneumococcus and its immunological reactions. Blood specimens are cultured in buffered dextrose veal infusion broth, containing 5 mg. per 100 ml. of *p*-aminobenzoic acid if the individual is undergoing sulfonamide therapy. A portion of sputum, washed in three changes of sterile saline and emulsified in saline, may be inoculated intraperitoneally in a white mouse. With virulent strains the animal will show signs of illness in 5 to 8 hours, and microscopic examination of smears of peritoneal exudate will show large numbers of encapsulated diplococci.

The pneumococcus is identified and typed with antiserum, usually by the Quellung reaction though agglutination and precipitin tests may be used also. When large numbers of the bacteria are present in sputum, the typing may be done directly without culture or mouse inoculation, but since the Quellung reaction is inhibited in the presence of large amounts of SSS, negative reactions are not significant. Typing is readily carried out, as indicated earlier, on pneumococci present in mouse peritoneal exudate or cultures but is no longer carried out except for special purposes, since type-specific serum therapy has been practically entirely supplanted by chemotherapy.

Chemotherapy. The pneumococci are uniformly susceptible to the sulfonamides and the commonly used antibiotics. The incidence of naturally occurring drug-resistant strains has been negligible, and determination of drug sensitivity is not yet an essential part of the chemotherapy of these infections. *In vitro* the pneumococci are approximately as sensitive to these drugs as is *Str. pyog-*

enes. In practice sulfonamides, sulfadiazine or sulfathiazole, and penicillin are the commonly used drugs. In pneumococcal meningitis, combined therapy with sulfonamide and penicillin has been reported to give better results than either alone; it has been reported that sulfonamide reduced the case fatality rate to 73 per cent, penicillin to 50 per cent and combined therapy to 29 per cent.

Pathogenicity for Lower Animals. The susceptibility of the usual laboratory animals to pneumococcus infection is variable, ranging from the highly susceptible mouse and rabbit through the less sensitive guinea pig to the cat, dog, chicken, and pigeon, which are highly resistant. Rare instances of naturally occurring infections in the usual experimental animals have been reported; for example, an epizootic of type 19 pneumococcus infection in the guinea pig has been described,[25] and one of type 2 infection in laboratory rats.[33] Animal experiments with the pneumococcus present an example of the general law that susceptibility is characterized by general septicemic infection, resistance by the occurrence of a localized process. The mouse and the rabbit develop rapidly fatal septicemia, and in these animals lung lesions, when they occur at all, are slight and usually limited to the bronchopneumonic type. It is possible to produce typical lobar pneumonia in the rabbit by carefully balancing the susceptibility of the animal and the virulence of the bacterium through the use of attenuated cultures or previous partial immunization. Resistant animals, such as the dog, show an approximation toward the type of pneumococcus infection observed in man, and lobar pneumonia may be produced in monkeys by intratracheal inoculation. The lesions produced in monkeys were considered identical with those in human lobar pneumonia. It is of some interest that pneumococci were found in the blood within 6 hours after their introduction into the trachea and before the signs of pneumonia appeared, suggesting the bronchogenic rather than the hematogenous origin of the infection.

Man, therefore, may be regarded as an animal of rather high normal resistance. This resistance may, however, be so reduced as to permit the production of localized manifestations, which in still more susceptible individuals may lead to fatal septicemia. In some cases death is due to overwhelming interference with respiration caused by the local pulmonary lesions; in others, to a general systemic poisoning or toxemia.[5]

Immunity. Experimental animals may be actively immunized against pneumococcus infection by the injection of vaccines of the smooth, virulent bacteria, although the immunity is not of long duration, *i.e.*, not more than a few months. The development of the immune state is accompanied by the appearance of antibodies, precipitins, agglutinins, and the like, as well as a protective quality in the blood serum. In man the situation is somewhat obscure; there is undoubtedly an intimate relation between recovery and the appearance of humoral antibodies, but immunity following infection is slight and transitory, and one attack may succeed another after a short interval. Active immunity in experimental animals is type-specific, however, and it is not improbable that under natural conditions different immunological types may participate in successive attacks.

The question of active immunization of man to pneumococcus infection is of continued interest. Results suggestive of its value were obtained from mass inoculation studies in 1918–1919 in this country. It has been shown[22] that the inoculation of type-specific polysaccharides induces an antibody response which reaches a peak within 6 weeks and persists for about 6 months; booster inoculation during the following 18 months was not effective in raising the diminishing titers. It has been found[32] also that such immunization significantly reduced both carrier rate and cases of infection with the pneumococcus types immunized against, though not the incidence

of other types. In the aggregate, then, these data suggest that an appreciable degree of effective immunity may be produced by immunization procedures.

The results of the therapeutic use of antiserum are variable, being excellent with some types of pneumococci and not with others. Serum therapy was formerly of great practical importance, but with the introduction of effective chemotherapeutic agents is no longer used.

REFERENCES

1. Adams, M. H., and Rose, A. S.: A partially defined medium for cultivation of pneumococcus. J. Bact., 1945, **49:**401–409.
2. Alexander, J. D., Jr., Flippin, H. F., and Eisenberg, G. M.: Pneumococcic meningitis. Study of one hundred two cases. Arch. Intern. Med., 1953, **91:**440–447.
3. Anderson, T.: Pneumonia—a survey—past and present. Edinburgh Med. J., 1948, **55:**705–720.
4. Austrian, R.: Morphologic variation in pneumococcus. I. An analysis of the bases for morphological variation in pneumococcus and description of a hitherto undefined morphologic type. J. Exptl. Med., 1953, **98:**21–34.
5. Austrian, R.: Morphologic variation in pneumococcus. II. Control of pneumococcal morphology through transformation reactions. J. Exptl. Med., 1953, **98:**35–40.
6. Austrian, R.: The prevalence of pneumococcal types and the continuing importance of pneumococcal infection. Amer. J. Med. Sci., 1959, **238:**133–139.
7. Austrian, R., Bernheimer, H. P., Smith, E. E. B., and Mills, G. T.: Simultaneous production of two capsular polysaccharides by pneumococcus. I. Properties of a pneumococcus manifesting binary capsulation. J. Exptl. Med., 1959, **110:**571–584.
8. Austrian, R., Bernheimer, H. P., Smith, E. E. B., and Mills, G. T.: Simultaneous production of two capsular polysaccharides by pneumococcus. II. The genetic and biochemical bases of binary capsulation. J. Exptl. Med., 1959, **110:**585–602.
9. Badger, E.: The structural specificity of choline for the growth of type III pneumococcus. J. Biol. Chem., 1944, **153:**183–191.
10. Badger, E.: The nutritional requirements of a strain of type III pneumococcus. J. Bact., 1944, **47:**509–518.
11. Bayliss, M.: A sodium lauryl sulfate solubility test for the identification of pneumococci. J. Lab. Clin. Med., 1943, **28:**748–751.
12. Eddy, B. E.: Nomenclature of pneumococcic types. Pub. Health Repts., 1944, **59:**449–451.
13. Eddy, B. E.: A study of cross reactions among the pneumococcic types and their application to the identification of types. Pub. Health Repts., 1944, **59:**451–468.
14. Eddy, B. E.: Cross reactions between the several pneumococcic types and their significance in the preparation of polyvalent antiserum. Pub. Health Repts., 1944, **59:**485–499.
15. Eddy, B. E.: A simplified procedure for detecting cross reactions in diagnostic antipneumococcic serum. Pub. Health Repts., 1944, **59:**1041–1045.
16. Forster, G. F., and Shaughnessy, H. J.: Occurrence of strains of pneumococci which react with more than one type-specific antipneumococcal serum. Proc. Soc. Exptl. Biol. Med., 1940, **44:**306–309.
17. Finland, M.: Recent advances in epidemiology of pneumococcal infections. Medicine, 1942, **21:**307–344.
18. Firshein, W.: DNA synthesis, respiration and virulence in pneumococci. Ann. N. Y. Acad. Sci., 1960, **88:**1054–1074.
19. Gilbert, E. B.: On the growth requirements of pneumococci. Proc. Soc. Exptl. Biol. Med., 1944, **57:**363–366.
20. Harris, A. H., and McClure, G. Y.: The use of a detergent in solubility tests for the identification of pneumococci. J. Lab. Clin. Med., 1942, **27:**1591–1592.
21. Heffron, R.: Pneumonia. With Special Reference to Pneumococcus Lobar Pneumonia. Commonwealth Fund, New York. 1939.
22. Heidelberger, M., MacLeod, C. M., Kaiser, S. J., and Robinson, B.: Antibody formation in volunteers following injection of pneumococci or their type-specific polysaccharide. J. Exptl. Med., 1946, **83:**303–320.
23. Heidelberger, M., and Rebers, P. A.: Immunochemistry of the pneumococcal types II, V, and VI. I. The relation of type VI to type II and other correlations between chemical constitution and precipitation in antisera to type VI. J. Bact., 1960, **80:**145–153.
24. Hodges, R. G., and MacLeod, C. M.: Epidemic pneumococcal pneumonia. V. Final consideration of the factors underlying the epidemic. Amer. J. Hyg., 1946, **44:**237–243.
25. Homburger, F., Wilcox, C., Barnes, M. W., and Finland, M.: An epizootic of pneumococcus type 19 infections in guinea pigs. Science, 1945, **102:**449–450.
26. Humphrey, J. H.: Hyaluronidase production by pneumococci. J. Path. Bact., 1944, **56:**273–275.
27. Julianelle, L. A., and Reimann, H. A.: The production of purpura by derivatives of pneumococcus. I. General considerations of the reaction. J. Exptl. Med., 1927, **43:**87–95.
28. Koenig, V. L., and Perrings, J. D.: Sedimentation and viscosity studies on the capsular and somatic polysaccharides of pneumococcus type III. J. Biophys. Biochem. Cytol., 1955, **1:**93–98.
29. Lund, E.: Four new pneumococcus types. Acta Path. Microbiol. Scand., 1950, **27:**720–725.
30. Lund, E.: The present status of the pneumococci, including three new pneumococcus types. Acta Path. Microbiol. Scand., 1957, **40:**425–435.
31. Lund, E.: Laboratory diagnosis of pneumococcus infections. Bull. W. H. O., 1960, **23:**5–13.
32. MacLeod, C. M., Hodges, R. G., Heidelberger, M., and Bernhard, W. G.: Prevention of pneumococcal pneumonia by immunization with specific capsular polysaccharide. J. Exptl. Med., 1945, **82:**445–465.
33. Mirick, G. S., et al.: An epizootic due to pneumococcus type II in laboratory rats. Amer. J. Hyg., 1950, **52:**48–53.

34. Mørch, E.: Pneumococcal hemolysin. Acta Path. Microbiol. Scand., 1946, **23:**555–575.
35. Oram, F.: Pneumococcus leucocidin. J. Immunol., 1934, **26:**233–246.
36. Pochon, J.: Recherches sur les enzymes proteolytiques du pneumocoque. Ann. Inst. Pasteur, 1942, **68:**81–83.
37. Rane, L., and Subbarow, Y.: Choline, pantothenic acid and nicotinic acid as essential growth factors for pneumococcus. J. Biol. Chem., 1940, **134:**455–456.
38. Rane, L., and Subbarow, Y.: Nutritional requirements of the pneumococcus. I. Growth factors for types I, II, V, VII and VIII. J. Bact., 1940, **40:**695–704.
39. Ruegsegger, J. M.: Pneumococcal meningitis. A review. U. S. Naval Med. Bull., 1949, **49:**1159–1168.
40. Rumreich, A. S., et al.: A nation-wide study of the bacterial etiology of the pneumonias. Pub. Health Repts., 1943, **58:**121–135.
41. Smillie, W. G., Calderone, F. A., and Onslow, J. M.: The epidemiology of the pneumococcus. The prevalence of pneumococci in the throats of a group of homeless men. Amer. J. Hyg., 1943, **37:**156–163.
42. Smillie, W. G., and Duerschner, D. R.: The epidemiology of terminal bronchopneumonia. I. The significance of post-mortem cultures in determination of the etiology of terminal pneumonia. Amer. J. Hyg., 1947, **45:**1–12.
42. Smillie, W. G., and Duerschner, D. R.: The epidemiology of terminal bronchopneumonia. II. The selectivity of nasopharyngeal bacteria in invasion of the lungs. Amer. J. Hyg., 1947, **45:**13–18.
44. Smillie, W. G., and Jewett, O. F.: The epidemiology of pneumonia. The role of type 14 pneumococci in producing illness. Amer. J. Pub. Health, 1942, **32:**987–995.
45. Spink, W. W., and Su, C. K.: Persistent menace of pneumococcal meningitis. J. Amer. Med. Assn., 1960, **173:**1545–1548.
46. Thompson, R. T., Ruegsegger, J. M., Blankenhorn, M. A., and Hamburger, M.: Primary pneumococcic pneumonia at the Cincinnati General Hospital 1936–1950. J. Lab. Clin. Med., 1951, **37:**73–87.
47. White, W. B., Jr., Smith, M. R., and Watson, B.: The Biology of Pneumococcus: The Bacteriological, Biochemical, and Immunological Characters and Activities of *Diplococcus pneumoniae*. Commonwealth Fund, New York. 1938.

Chapter 19

THE GRAM-NEGATIVE PATHOGENIC COCCI (NEISSERIA)

The Gonococcus and the Meningococcus

THE GONOCOCCUS and the meningococcus are the chief representatives of a small group of closely related bacteria whose other members are nonpathogenic inhabitants of the mouth and upper respiratory tract of man.[66] Two groups of species are separated from one another on the basis of pigment production, and further differentiation is made by means of fermentation reactions. Pigmented varieties are frequently found in the nasopharynx (*Neisseria flava* I, II, III).

THE GONOCOCCUS

Neisser in 1879 first called attention to the constant presence of a peculiar coccus in gonorrheal pus. In cases of gonorrhea of recent origin this was the sole organism found; it not only occurred in the urethral and vaginal discharges of ordinary gonorrhea, but was present in the exudate in conjunctivitis due to gonorrheal infection. Pure cultures of this microorganism were isolated in 1885 by Bumm, who succeeded in demonstrating its etiological relation to gonorrhea by the inoculation of human volunteers. This bacterium, known generally as the *gonococcus*, has been termed *Micrococcus gonorrheae* and *Diplococcus gonorrheae*, but the genus Neisseria is now more or less generally accepted, and this bacterium is properly known as *N. gonorrhoeae*.

Morphology and Staining. In preparations made from gonorrheal pus the cells of the gonococcus occur in pairs, with the flattened sides in juxtaposition; the appearance in stained preparations resembles that of a coffee bean. In pure culture the cocci appear as oval or spherical and are often aggregated in irregular masses without the typical diplococcus arrangement. In pus smears the gonococcus occurs almost entirely within the leucocytes; frequently enormous numbers may be found packed within a single phagocyte. In the earliest stages of infection gonococci may be found extracellularly, and the same is true of cases of gonorrhea of long standing. The gonococcus is nonmotile and does not form spores.

The colonies of the gonococcus are

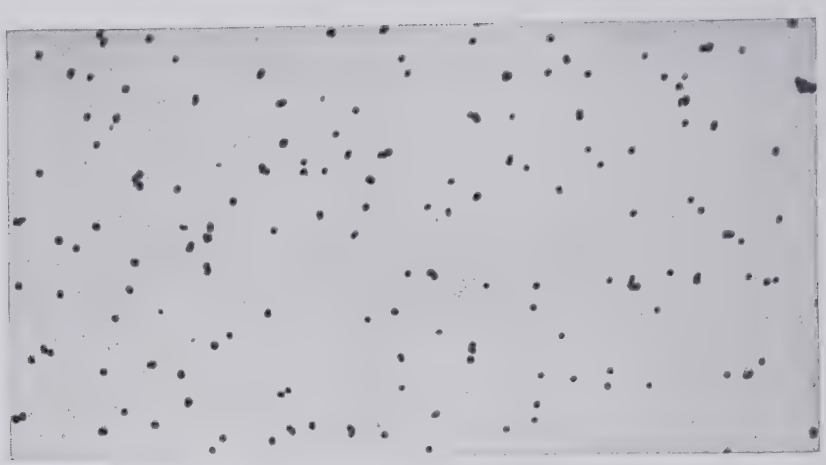


Figure 91. The gonococcus from pure culture. Fuchsin; × 1050.

Fermentation Reactions of the Gram-Negative Diplococci

NON-PIGMENTED SPECIES	DEXTROSE	MALTOSE	SUCROSE	LEVULOSE	MANNITOL
N. gonorrheae (gonococcus)	+	−	−	−	−
N. intracellularis (meningococcus)	+	+	−	−	−
N. catarrhalis	−	−	−	−	−
N. sicca	+	+	+	+	−
PIGMENTED SPECIES					
N. perflava (*flava I*)	+	+	+	+	+
N. flava (*flava II*)	+	+	−	+	−
N. subflava (*flava III*)	+	+	−	−	−
N. flavescens	−	−	−	−	−

small, translucent, finely granular with lobate margin and grayish white with a pearly opalescence when viewed by transmitted light. Larger colonies may be formed on special mediums. Colonial appearance is, however, subject to variation (see below).

Unlike the other pyogenic cocci, the gonococcus and related forms are gram-negative, a staining characteristic that is of considerable diagnostic value since it serves to differentiate the gonococcus from other cocci present in the urethral or vulvovaginal tracts. Other gram-negative cocci may be found occasionally, sometimes within the leucocytes, but they are rare. The tendency to decolorization in the Gram stain is variable. Some strains decolorize much more readily than others, and gonococci embedded in masses of pus may retain the stain; hence the preparation of thin, uniform films is highly desirable. The gonococcus stains with the aniline dyes, but polychrome stains, such as Pappenheim's stain,[4] are more useful. Intracellular granules may be found in stained preparations, but in general the gonococci from young cultures stain evenly, while older cultures (24 hours and older) contain large swollen involution forms, which may stain poorly.

Physiology. In its nutritive requirements the gonococcus is one of the most fastidious bacteria, particularly upon primary isolation, and an enriched medium is required for cultivation. Earlier mediums were enriched by the addition of ascitic and hydrocele fluid. Chocolate (heated blood) agar has been the most widely used, and the basal medium may be enriched with horse plasma and hemoglobin, and sometimes Nile blue A is added.

The nutritional requirements of the gonococcus are complex although some

strains may be grown on a tryptic digest–glucose agar containing added cystine, and relatively complex synthetic mediums have been devised which will support growth.[27, 30, 40] Stock strains are commonly less exacting than recently isolated strains, and it is likely that growth requirements are more accurately reflected in the mediums required for isolation or growth of fresh isolates. Such strains do appear to require added glutamine and cocarboxylase since they apparently cannot phosphorylate thiamin, and glutathione is required by some strains.

A sufficient supply of moisture is essential; there should be water of condensation on the tubes or plates, and the atmosphere of the incubator should be kept saturated with water. Incubation in an atmosphere of increased CO_2 tension, about 10 per cent, greatly improves growth and is a practical necessity in primary isolation.[29]

With continued cultivation on laboratory mediums, the gonococcus appears to become somewhat less fastidious, and some strains may eventually grow upon the ordinary infusion mediums. The preservation of cultures is difficult, however, for the gonococci die off in 2 to 3 days at room temperature and in 6 to 8 days at 37° C. but will live longer when kept in the cold. Even upon continued transfer the gonococci die off, and cultures are often lost. The optimum temperature for growth is 37° C., growth does not occur below 30° C., and temperatures of 40° to 41° C. are definitely harmful. The gonococcus will grow under anaerobic conditions but is essentially aerobic in character.

With respect to deleterious influences the gonococcus is a delicate microorganism. It is readily killed by heat, as indicated above, and by dilute antiseptics; 1 per cent phenol, for example, kills in one to three minutes. It is remarkably sensitive to certain of the flavine dyes and is rapidly destroyed by silver salts. The gonococcus is sensitive to drying and, under ordinary conditions, can survive exposure to the air for only a very short time—1 to 2 hours—although in masses of dried pus it may live exceptionally for 6 to 7 weeks. Unlike most gram-negative bacteria, it is sensitive to penicillin as well as to streptomycin and the tetracyclines.

The gonococcus is not very active biochemically. Glucose is fermented, principally to lactic acid, but many other sugars are not attacked, indol is not produced, nitrates are not reduced, and no change is produced in litmus milk. Its fermentation of glucose and failure to ferment maltose distinguish it from the other nonpigmented Neisseria; the fermentation reactions are reliable and used for purposes of identification.[51] Catalase

Figure 92. Colonies of the gonococcus on blood agar. × 6.

is produced, and a characteristic that has been turned to practical differential use is the formation of indophenol oxidase. The specimen is cultured on 10 per cent heated blood (chocolate) agar in an atmosphere containing 8 per cent carbon dioxide, followed by 24 hours' incubation in air. A 1 per cent solution of tetramethyl-*p*-phenylenediamine is poured on the incubated plate and poured off again immediately or sprayed on with a nasal atomizer. Colonies of bacteria forming indophenol oxidase turn a bright purple color. The bacteria are not immediately killed and may be subcultured within half an hour. This so-called oxidase reaction, coupled with the examination of smears for gram-negative intracellular diplococci, is generally used in the laboratory diagnosis of gonorrhea.

Toxins. Other than a weak hemolysin, the gonococci apparently form no extracellular toxic substances, but the cell substance is toxic to experimental animals on parenteral inoculation and has been reported to produce suppuration when instilled into the human urethra. The toxicity is extractable in dilute alkali as a "nucleoprotein," and from this material or from the intact bacteria by trichloracetic acid or diethylene glycol,[7] in the latter instance behaving like a Boivin antigen, *e.g.*, the endotoxin of the gram-negative bacilli. It was subsequently found to be extracted in phenol by the Westphal method (p. 308) to give a toxic lipopolysaccharide which could be purified by fractional precipitation with acetone.[72, 73] This substance is similar to the gram-negative bacillus endotoxins as typified by colon bacillus endotoxin, but on analysis has been found to differ significantly in lacking diaminopimelic acid and glycine and in lesser content of other amino compounds.[74] The contained carbohydrate has been found to consist of D-glucosamine, glucose, and galactose, the amino acids presumably serving to link the sugar and lipid components in the intact molecule. The pharmacological activity of the gonococcal endotoxin appears to be nonspecific and overtly similar to that of the other endotoxins. Its role in the pathogenesis of gonococcal infections is uncertain.

Variation. Numerous workers have observed that the cultural characteristics of the gonococcus are subject to considerable variation. It has been found that two types of colonies may be observed which appear to be correlated with immunological type. The one, designated as type I, is a large, irregular, flattened, translucent colony that, on continued incubation, gives rise to surface papillae; the other type of colony, type II, is somewhat smaller, round, raised with slightly convex uneven

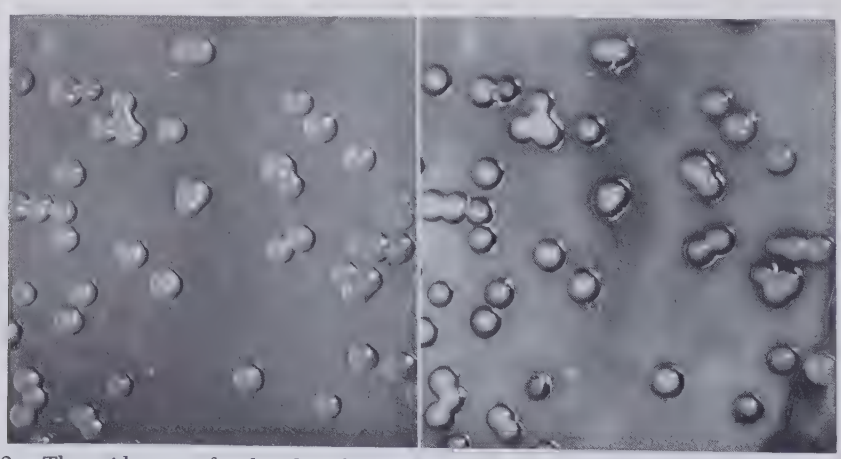


Figure 93. The oxidase test for the identification of gonococcus colonies. Pure culture on blood agar. *Left*, gonococcus colonies before the application of tetramethyl-*p*-phenylenediamine solution. *Right*, the same colonies after the application of the reagent. Note the greater intensity of color about the edges of the colonies immediately after application and the discoloration of the medium. × 5.

surface, and opaque. Type I colonies are generally recovered from acute gonorrhea, while type II are found in old laboratory strains and, sometimes, in chronic gonorrhea. The papillae are thought to represent the first step in transition toward the type II form. The relation of these forms to the dissociative changes of other bacteria is uncertain. Small colony variants, presumably arising from a dissociative process, have been produced experimentally and have also been found on primary isolation from clinical material.[50]

Drug resistance. Resistance of gonococci to the sulfonamides and antibiotics is readily acquired under experimental conditions, and resistant strains appear in nature coincident with the general application of chemotherapeutic agents. When the sulfonamides were introduced into general use as chemotherapeutic agents in 1936–37, 80 to 90 per cent of infections could be cured with them. An increase in the proportion of refractory infections became noticeable in three or four years, a sharp increase occurred in 1942–43 from 25–35 per cent to 50 per cent, and by the late 1940's as few as 15 per cent of infections in some areas responded to sulfonamide therapy.[81] Gonococcus strains isolated from refractory infections usually prove to be resistant on laboratory examination, many, but not all, of them producing *p*-aminobenzoic acid in excess amounts.

Meantime penicillin therapy became available in the middle 1940's and has been widely used, usually as repository penicillin, except in France where streptomycin has been preferred to avoid masking of concurrent syphilitic infection. Subsequently other antibiotics, the tetracyclines, erythromycin, chloramphenicol, etc., effective in the treatment of gonorrhea, became available also but have not been generally used. Strains of gonococci resistant to penicillin did not appear in increased numbers until about 10 years after its introduction, but by the late 1950's it was apparent that 20 per cent or more of strains isolated in many areas were resistant to high concentrations, twentyfold or more the original sensitivity, not ordinarily achieved as blood levels.[11, 13, 45, 63, 75] A temporary, and effective, expedient has been the use of larger doses of the antibiotic. Concurrently, strains of gonococci resistant to streptomycin have increased in number in France, with 75 per cent of strains resistant to 50 μg/ml. or more, and more than 20 per cent resistant to 1000 μg/ml., in contrast to an original sensitivity level of perhaps 5–10 μg/ml.[22, 64]

Increase in the proportion of resistant strains coincides with the use of the chemotherapeutic agent. There seems to have been no significant increase in penicillin-resistant strains of gonococci in France, nor an increase in streptomycin-resistant strains in other European countries and the United States where penicillin has been used. The tendency to reversion to sensitivity when the chemotherapeutic agent is discontinued, apparent in some other bacteria, *e.g.*, the staphylococci, is indicated by the decline in the proportion of sulfonamide-resistant strains, which by 1958 reached approximately the level which prevailed when sulfonamide therapy was generally introduced.[16]

Clinical refractoriness to penicillin therapy is not necessarily always a consequence of infection with a resistant strain of gonococcus. Within the past two decades the incidence of nongonococcal urethritis has increased.[2, 34, 54, 55] Further, some cases may be refractory because of failure of the antibiotic to affect phagocytosed gonococci which have been shown experimentally to survive considerable increases in concentration of penicillin over the minimal inhibitory concentrations.[76]

Antigenic Structure. The gonococci are apparently of uncertain antigenic structure as assayed by conventional methods of antigenic analysis.[60, 61, 62] Certain of the antigens appear to be thermostable, and thermostable and thermolabile partial antigens occur also, some of which may function as blocking antigens, and antigenicities appear to be shared with meningococcus and some strains of Pasteurella. Gonococcus strains

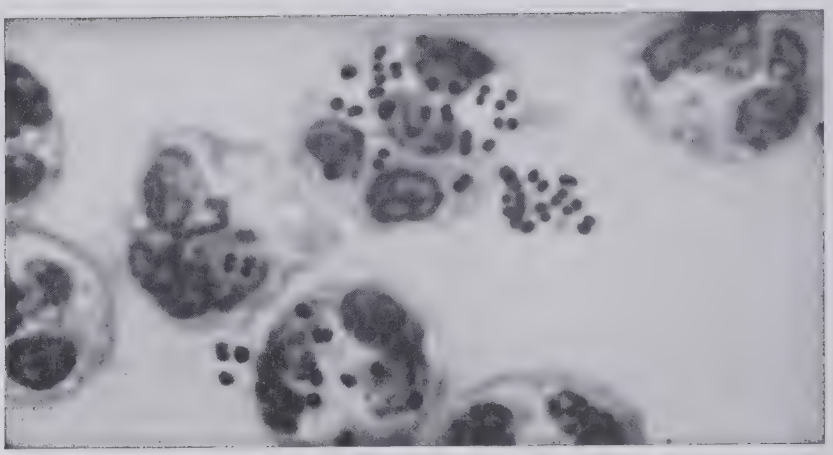


Figure 94. Urethral smear from gonorrhea. Gram stain. Note the intracellular and extracellular position of the gonococci and their typical coffee-bean shape and arrangement in pairs. ×2400.

not only differ when freshly isolated, but also apparently change antigenically on continued subculture. Studies with soluble antigen, prepared by extraction with dilute alkali and precipitation with ethanol from neutral solution, in the passive hemolysis reaction have given more consistent results.[14] Two main antigens are demonstrable by this technique, the one associated with colonial type I which is shared with meningococcus, and the other with type II. The dual nature of the antigenicity is also indicated by differentiation of the antigenicity which sensitizes red cells to immune lysis in the presence of antiserum from that which fixes complement. The relation of this antigenicity to the lipopolysaccharide endotoxin has not yet been described, but it is heat-stable and presumably polysaccharide in nature. Antiserum to the alkali-extracted endotoxin described above is protective in the mouse.[7]

Further study has shown that the heat-stable antigenic complex contains at least six antigens shared with the meningococcus, and which occur in varied combinations,[84] and also that freshly isolated strains of gonococcus are often inagglutinable in antiserums to these antigens.[83] The last has been taken to suggest that fresh isolates may contain a K-type antigen (Chap. 20), labile in that it is readily lost, and functioning as a blocking antigen. The existence of this kind of antigen in the gonococcus has been demonstrated by the fluorescent antibody technique.[19] It appears to be sharply specific for the gonococcus, and is present in relatively large amount on gonococci in acute infections, *i.e.*, as observed in urethral smears, and in very young cultures, but has largely disappeared in 30 hour cultures.

Pathogenicity for Man.[33] Few diseases are so widely disseminated through all classes of society as gonorrhea. Precise information as to the incidence of the disease is not available. It is estimated that in the United States no more than 10 to 20 per cent of cases are reported, and that gonorrhea is the third most common infection (after measles and streptococcal infections).[32] In 1944 it was predicted that, with the means, penicillin, available, the virtual eradication of gonorrhea could be anticipated. In that year 301,000 cases were reported, and the number rose to 401,000 in 1947, subsequently declining somewhat to stabilize, since 1952, at about 220,000 cases reported per year. More than half of these are persons under 25 years, with a modal age of 18 for females and 22 for males.[11]

The gonococcus usually gains entrance to the tissues after deposition on the surface by burrowing through the superficial epithelium, between the cells, to reach subepithelial connective tissue from which it spreads by direct continu-

ity or by the lymphatic or blood vessels. Stratified squamous epithelium is more resistant to this penetration than columnar epithelium. The characteristic reaction within the tissues is a dense cellular infiltration of polymorphonuclear leucocytes, plasma and mast cells, and these are eventually replaced by fibrous tissue. In the male, epididymitis, chronic urethritis and stricture and other inflammatory conditions develop, and in the female, the entire genitourinary tract may be involved, and the fallopian tubes, the ovaries, and the peritoneum are not uncommonly invaded. The gonococcus may also invade the blood stream from local lesions and be carried to various parts of the body to give rise to a variety of extragenital lesions. Especial predilection is shown for the synovial membranes of the joints, where it causes the so-called gonorrheal rheumatism, and for the heart valves, where it produces endocarditis. Local or general complications occur in perhaps 30 per cent of all cases. Gonococcal meningitis occurs, perhaps more frequently than formerly thought. There has been a general belief that the incidence of complications, epididymitis, prostatitis, salpingitis and arthritis, has been less since the introduction of effective chemotherapy, but this appears to be open to question.[26]

Once established, untreated gonococcal infection persists for a long time; 5 to 15 years' duration has been reported. Exclusion of reinfection is a difficult matter, but infection of 7 years' duration without reinfection has been observed. Subclinical infection may occur, with or without transient symptoms, and individuals so infected may act as carriers.[5]

Gonorrheal vulvovaginitis occurs in epidemic form in little girls, and in these instances the infection is transmitted by bedclothes, towels, common bathtubs, and other fomites. The commonest complications are urethritis, proctitis, and cervicitis. Such epidemics are frequently exceedingly difficult to control and constitute a serious problem in many institutions such as children's wards in hospitals.[15, 71]

Gonorrheal ophthalmia of the newborn is a well-known consequence of maternal infection; infection does not occur *in utero* but during passage through the birth canal. Although exact information is not obtainable, it is estimated that 10 per cent of all cases of blindness are traceable to this source[25] and that in the United States there are perhaps 12,000 children blind from this cause. Infection may be prevented by the prophylactic instillation of silver salts such as silver nitrate. Some of the antibiotics,[43] especially penicillin, are equally effective and may be given by the intramuscular route.[18]

Except in the case of vulvovaginitis in children, gonorrhea is spread by direct contact, usually sexual. Once infected, an individual may remain infective for a long time, and gonococci may persist in the genitourinary secretions for years after apparently complete recovery, and even though they may not be found by bacteriological examination the infection may be transmitted. Gonorrhea persists, then, in the human population in a smoldering endemic form and, as a consequence of the nature of its transmission, widespread epidemics in the usual sense do not occur.[82]

Bacteriological Diagnosis of Gonococcus Infection.[39, 56, 59] The demonstration and identification of the gonococcus are essential to the diagnosis of gonorrhea, and it should be borne in mind that every case of gonorrhea is potentially a medico-legal case. The presence of gram-negative intracellular diplococci in urethral smears in most cases justifies a provisional diagnosis of gonorrhea. The fluorescent antibody method[19] is a promising method of specific identification of the gonococcus in such smears. As a rule gonococci may be found readily in acute infections, but in chronic infections, especially in the female, demonstration of the organisms by direct smear or culture is usually difficult. Morphologically similar bacteria may, however, be present in the vagina or conjunctiva. Of forty-three such cultures, Johnston[37] found thirty-five were *N. sicca* and twelve were *N. subflava,*

and Wilkinson[80] has observed an incidence of nongonococcal Neisseria of 3.4 per cent in women and 1.5 per cent in men. Nongonococcal urethritis sometimes occurs more often than gonorrhea, *viz.*, a ratio of 3:1 in the United States Navy has been reported and is apparently increasing both relatively and absolutely.[2] There is some evidence that at least a portion of these are caused by nonsporulating obligate anaerobes of the pleuropneumonia group. The suggestion that these may represent L forms or protoplasts of the gonococcus appears to be unfounded.[65]

It is generally agreed that culture of the gonococcus gives a higher proportion of positive results than direct smear examination alone, and cultured gonococci may be identified. The question of survival of this relatively delicate bacterium in transport of specimens is of some importance. Stuart's transport medium[70] is considered to be by far the most satisfactory. For culture an infusion chocolate agar, developed by McLeod, or minor modifications of it such as the plasma hemoglobin modification studied by Thayer, Schubert, and Bucca[77] is a medium of choice, and is inoculated directly with the specimen (or sediment if it is urine or spinal fluid). The culture must be incubated in 10 per cent CO_2; this atmosphere may be satisfactorily approximated by putting the plates in a jar together with a lighted candle and sealing or by the inclusion of a handful of moistened fresh oats in a sealed container with the cultures. The oxidase test serves to differentiate the oxidase-positive colonies and, if picked immediately, they may be subcultured. Identification is based upon sugar fermentations in serum broth or serum agar containing an indicator.

Chemotherapy. As described above, the occurrence of resistance to sulfonamides and antibiotics markedly affects successful chemotherapy. Disregarding such resistance, the gonococci are sensitive to sulfonamides, usually sulfadiazine, and to the antibiotics penicillin, erythromycin, tetracyclines, chloramphenicol, carbomycin, streptomycin, neomycin, and bacitracin in that order, on the basis of activity per unit weight.[20] Since streptomycin, which occurs far down on the list, is effective often in a single dose, there is a variety of available chemotherapeutic agents.

Pathogenicity for Lower Animals. The gonococcus is nonpathogenic for lower animals, aside from the toxicity of the cell substance as noted above, and gonorrhea has never been reproduced in experimental animals, including anthropoid apes. An experimental infection of the anterior chamber of the rabbit's eye has been described by Miller and his co-workers[21, 47, 48] in which the gonococci multiply and invade intraocular tissues, especially the ciliary body and lens, giving rise to a chronic infection in approximately one-third of the animals. This infection has been made use of in the study of the efficacy of chemotherapeutic agents.

Immunity. Little if any immunity to the gonococcus is acquired as a result of infection, and second and third infections may be superimposed upon the first, *i.e.*, acute upon old chronic infections. As might be expected, then, the therapeutic use of vaccines and various types of antiserums is without effect. The significance of the observed extensive phagocytosis of gonococci by polymorphonuclear leucocytes is uncertain.

Some degree of immunological response is evident, however. Complement-fixing antibodies are usually present, and patients may give a marked skin reaction to suspensions of killed gonococci. A number of attempts have been made to utilize these responses in the immunological diagnosis of gonorrhea. The complement-fixation test has shown some promise but is not generally used. The skin reaction is apparently too variable to have practical value. It has also been observed that the discharges from gonorrheal inflammation give a precipitin reaction with antigonococcus serum, but this flocculation reaction has as yet no diagnostic value.

THE MENINGOCOCCUS[8, 52]

Inflammation of the meninges or investing membranes (pia-arachnoid) of the brain and spinal cord may be provoked by a variety of microorganisms and may occur either as a primary affection or secondarily in the train of an infection originally begun elsewhere. One form of meningitis, characterized especially by epidemic spread and usually designated as *epidemic cerebrospinal meningitis*, spotted fever, or cerebrospinal fever, is caused by a specific microorganism commonly known as the meningococcus.

This bacterium was described by Marchiafava and Celli in the meningeal exudate as early as 1884, but the first important work upon it was that of Weichselbaum, who, in 1887, obtained it in pure culture and described it in detail as the characteristic micrococcus found in six cases of acute cerebrospinal meningitis. Confirmation was supplied by the work of Jäger in spite of some faulty observation.

The meningococcus has been designated by a variety of names, including *Micrococcus meningitidis, M. intracellularis meningitidis, Neisseria intracellularis*, and, according to Bergey, *N. meningitidis*. Although Neisseria is generally accepted, common usage is divided between *N. intracellularis* and *N. meningitidis*.

Morphology and Staining. In film preparations of the meningeal exudate the meningococcus is very like the gonococcus and occurs in pairs or tetrads both within the leucocytes and free. The diplococci are flattened toward one another like gonococci, and there is considerable variability in the size of different cells in the same smear. In cultures the meningococcus averages a little less than 1 μ in diameter and appears, as a rule, in pairs; short chains are seen more rarely. The variability in size observed in meningeal exudate may also be seen in cultures, particularly in those more than 24 hours old. Involution forms are common, and it is not unlikely that the larger cells are degenerative. Capsules are usually not apparent but become swollen in the presence of specific immune serum—the Quellung reaction. The meningococcus is nonmotile and does not form spores.

Meningococcus colonies in blood agar are moist, elevated, smooth, and with a bluish-gray tinge. They do not produce green discoloration or hemolysis and may be readily differentiated from the hemolytic and viridans streptococci and the pneumococcus. The colonies are not so white and opaque as those of the staphylococci.

The meningococcus stains readily with the usual aniline dyes and, like the gono-

Figure 95. Meningococcus, pure culture. Note the typical diplococcus arrangement. Fuchsin; × 1050.

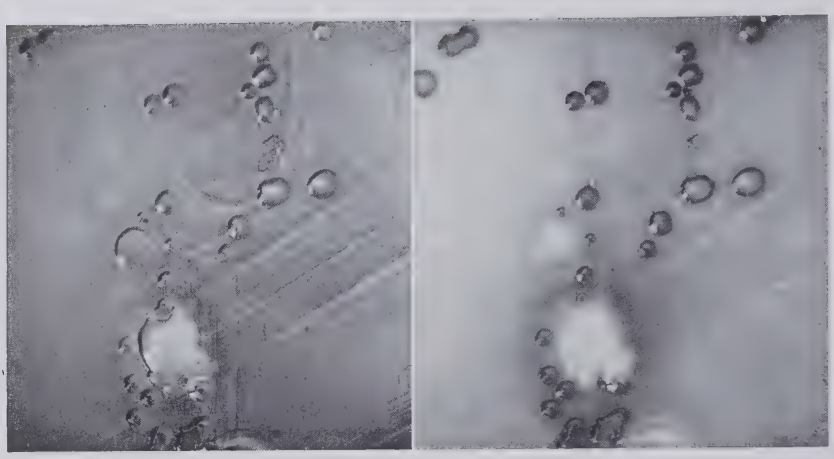


Figure 96. The oxidase test for the identification of meningococcus colonies. Mixed culture on blood agar. *Left,* colonies of meningococci and contaminants before the application of tetramethyl-*p*-phenylenediamine solution. *Right,* the same colonies after the application of the reagent. Note that the meningococcus colonies show the development of color first about the edges, and there is slight discoloration of the medium. × 5.

coccus, is gram-negative. The involution forms found in cultures tend to stain unevenly but even young cells may show the presence of metachromatic granules when stained by Löffler's alkaline methylene blue and other stains, and to a greater extent than the gonococcus. No sure distinction between the meningococcus and the gonococcus can be made on morphological grounds, and the identification of gonococci in gonococcal meningitis is dependent upon culture and differential fermentations.

Physiology. Strains of meningococci vary considerably in the ease with which they may be cultivated; some strains will grow, although sparsely, on nutrient and infusion mediums, but in general rich mediums containing serum or whole blood are required. Infusion base chocolate agar and blood agar are the most useful mediums. The nutritive requirements of meningococcus are similar to those of gonococcus, but some strains do not seem to require added cystine. Stock strains have been grown on synthetic mediums containing glutamic acid and cystine, or glutamic acid and lactate or glucose, and occasional freshly isolated strains will grow on these mediums.[6, 31, 67]

In isolating the meningococcus it is essential that the culture medium be warm when inoculated and kept warm until finally placed in the incubator. Growth is favored, especially in primary isolation, by incubation in an atmosphere containing 10 per cent carbon dioxide. The meningococcus will grow over a temperature range of 25° to 42° C. with an optimum at 37° C. Although sparse growth will occur under anaerobic conditions, the meningococcus is, for all practical purposes, an aerobe.

Continued cultivation on laboratory mediums results in more luxuriant growth, and the bacteria presumably become less nutritionally fastidious. Meningococcus cultures are difficult to keep, however, and tend to die out in stock culture. In most mediums these bacteria die within a few days if not transferred, but vitality may be preserved for several weeks in stab cultures in starch agar (1 per cent cornstarch in nutrient agar) and are best kept in the incubator.

The relatively early appearance of involution forms in meningococcus cultures as well as their limited viability when not transplanted is, perhaps, attributable to their formation of an active autolysin, and in saline suspension in the incubator autolysis may take place within a few hours. The autolysin is heat-labile, being destroyed at 65° C. in 30 minutes, and suspensions prepared for agglutination studies should be inactivated in this way.

The meningococcus, like the gonococcus, is a delicate microorganism and not highly resistant to deleterious influences. It is killed in a short time by drying and by exposure to dilute disinfectants. It is particularly sensitive to heat and cold and, unlike many bacteria, dies out within a few days at 0° C.

The meningococcus is not an active fermenter. Considerable quantities of acid, presumably lactic for the most part, are formed from glucose and maltose. The fermentation of maltose serves to distinguish the meningococcus from the gonococcus. Neither is this bacterium actively proteolytic, for coagulated serum is not liquefied.

Toxins. Meningococcus meningitis in man and that reproduced in experimental animals are usually accompanied by profound toxemia. The meningococcus, however, appears to form no soluble toxin, though its cell substance is toxic to experimental animals when injected in relatively large amounts. The toxicity is heat-stable (100° C. for 30 minutes) and the rate of its destruction suggests that the endotoxin consists of two substances, one much more thermostable than the other.

Variation. Rough and smooth colony types of the meningococcus have been described. Recently isolated strains are generally smooth while old stock cultures are rough. Mucoid colonies are observed. The change from smooth to rough is associated with a partial loss of immunological type specificity.

Classification. The meningococci are closely related to the gonococci, not only morphologically and physiologically but immunologically in that certain antigenic substances appear to be held in common as indicated previously.[84]

The meningococci are not antigenically homogeneous, and occur as several serotypes, demonstrable by the agglutination and Quellung reactions,[49] which are determined by polysaccharide haptenes except one, the P substance, which is a toxic protein. The polysaccharides of type I (type A)[38] and type C (type IIα)[78, 79] have been prepared in purified form, and the latter has been found to contain sialic acid and hexosamine. The relationship of the P substance to the endotoxins is not known. Differentiable types have been described by a number of workers since 1914, using different systems of notation. This has resulted in some confusion, and an internationally acceptable recommendation in which the serotypes are designated types A, B, C, and D has been agreed upon.[10] The

The Nomenclature of Meningococcus Types*

DOPTER AND PAURON, 1914	WOLLSTEIN, 1914	GORDON AND MURRAY, 1915	GRIFFITH AND SCOTT, 1916	PULLON, 1917	NICOLLE, DE BAINS AND JOUAN, 1918	EVANS, 1920	COMMON USE SINCE 1940	RECOMMENDED†
Meningococcus	Normal	I	I	C	A	R	I	A
	Irregular	III		A				
Parameningococcus	Parameningococcus	II	II	B	B	S	II	B
α, β, γ		IV	II		B	Z	IV	D
					C		IIα	C
					D‡			

* From Branham.[10]

† By the subcommittee on the taxonomy and nomenclature of Neisseria of the Nomenclature Committee of the International Association of Microbiologists.

‡ The relation of this type to the others is unknown.

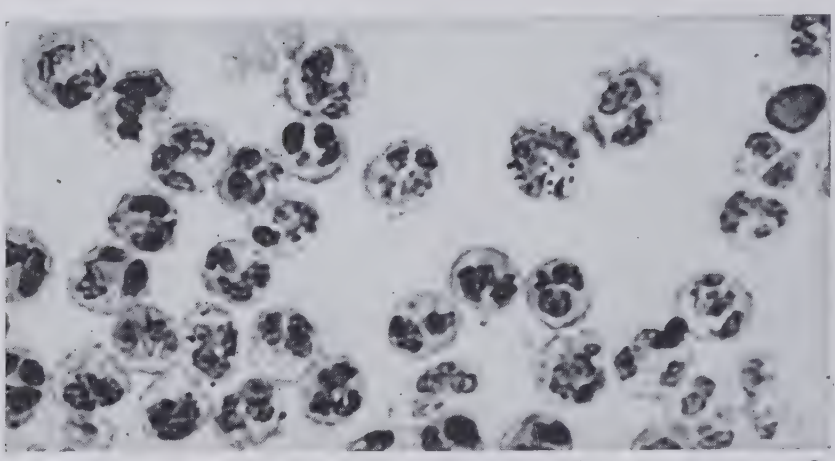


Figure 97. Meningococci in spinal fluid, showing phagocytosis of the microorganisms. Gram stain; × 1050.

various systems of nomenclature are illustrated in the accompanying table.

While the routine typing of meningococci is probably not worth while, typing in connection with epidemiological studies, *i.e.*, the distribution of types occurring in healthy carriers as well as in cases of the disease, has been valuable.

Pathogenicity for Man.[3, 23, 41] The resistance of man to meningococcus infection is relatively high, and the incidence of healthy carriers is invariably considerably higher than that of cases of the disease. It is probable that predisposing factors play a large part in determining whether or not infection will occur; insufficient clothing, inadequate ventilation, exposure to inclement weather, and fatigue very likely contribute in large measure to increasing susceptibility.

The meningococcus is initially present in the nasopharynx and from there gains access to the central nervous system. The route by which this occurs is uncertain; it is thought by some that the bacteria follow the perineural spaces of the olfactory nerves or set up a preliminary sinusitis and reach the brain either via the lymphatics or by direct extension through the bone. Others believe the meningococci reach the central nervous system via the blood stream through a preliminary bacteremia. While there is no definitive evidence concerning the means by which meningococci reach the central nervous system from the nasopharynx, the evidence appears to favor the hematogenous route. Occasionally the infection in the nasopharynx may extend into adjacent areas, giving rise to conjunctivitis, pneumonia, or other infections.

In the healthy carrier the infection remains confined to the nasopharynx and in this case is short-lived there and produces few or no symptoms. When the blood stream is invaded early in the disease hemorrhages usually occur in the skin and petechiae appear, especially on the wrists and ankles, or on any mucous or serous surfaces. These are apparent in 24 hours after invasion and fade in a few days. The rash is quite different from other purpuric rashes; the spots take geometrical shapes and are highly irregular in size. Meningococci may be observed in smears of material taken from these lesions. Other symptoms include sudden onset, chills, fever, and meningeal symptoms such as headache and drowsiness. Pain in the arms and legs is common. The invasion of the blood stream may take the form of a fulminating meningococcemia (the Waterhouse-Friderichsen syndrome) with adrenal apoplexy as the immediate cause of death and massive bilateral hemorrhage of the adrenals as the outstanding pathology.[36, 42, 53] This form of meningococcal disease is uncommon—little more than 200 cases have been reported—and

occurs much more frequently in infants than in adults. Its sudden and violent character with rapid fatal termination has led to deaths due to this cause being classified as suspicious. The bacteremia may also take a more chronic form, and give rise to a purulent synovitis or meningococcal arthritis.[12, 17]

Upon reaching the central nervous system, the meningococcus sets up a suppurative lesion of the meninges which involves the surface of the spinal cord together with the base and cortex of the brain. The spinal cord lesions are of two main types during the acute phase of the disease; one is an acute transverse myelitis, and the other a poliomyelitic type. Later there is a diffuse arachnoiditis that constricts the cord and interferes with its blood supply and may result in extensive intramedullary changes. The microorganisms are invariably present in the spinal fluid, which may vary from a slight to a heavy turbidity. The bacteria are found, sometimes in great numbers, both free and within the leucocytes in smears of spinal fluid. The case fatality is variable, but in any case high, and ranges from 35 per cent to 80 per cent and has been reduced to 16 per cent by improved methods of treatment, including chemotherapy.

There is some divergence of opinion regarding the nature and extent of sequelae to meningococcus meningitis, but these appear to include deafness, which is the most common, blindness, and pain and weakness of the neck, arms, and legs. In a representative series of cases, for instance, 7 to 8 per cent of the patients showed sequelae persisting for 9 to 30 months after recovery.

Epidemiology.[24, 28, 35, 85] Like most of the respiratory infections, meningococcus meningitis is disseminated by direct contact and droplet infection through the secretions of the mouth, nose, and throat. Infection is spread by patients and convalescents to a limited extent, but healthy carriers of meningococci are of primary importance.[57, 58] Some persons are temporary carriers, while others are chronic, discharging meningococci more or less continuously or in a sporadic fashion. Of a group of ten carriers, half were of the chronic type, carrying what was apparently the same strain of microorganism for over 2 years. Weekly

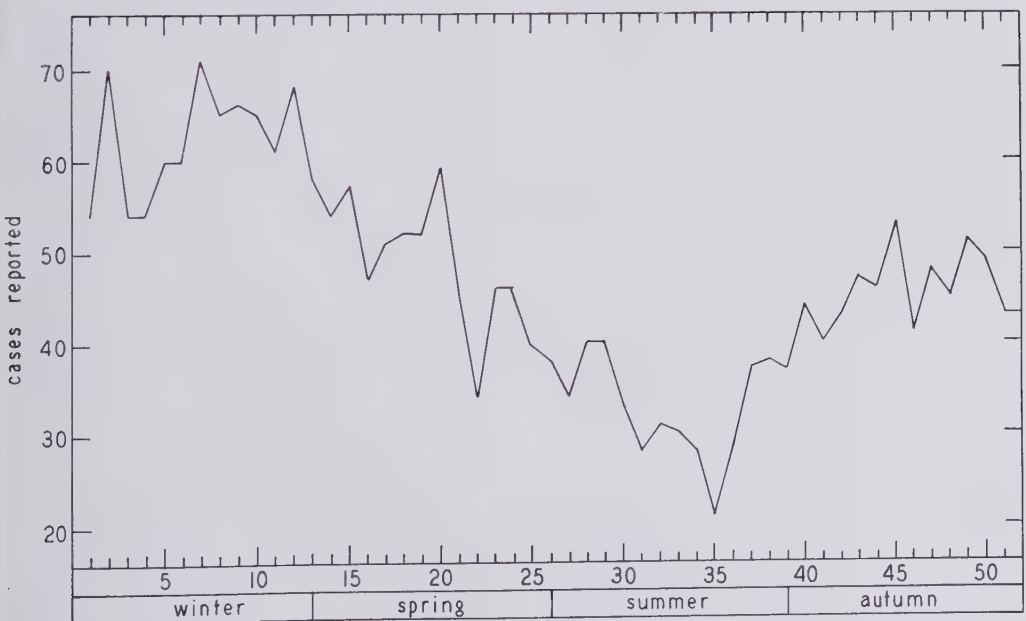


Figure 98. The seasonal incidence of meningococcal infections shown by the 5-year median number of cases reported weekly, 1956–60. Data from the Morbidity and Mortality Weekly Report, vol. 9, 1961. Communicable Disease Center, Public Health Service, U. S. Department of Health, Education and Welfare.

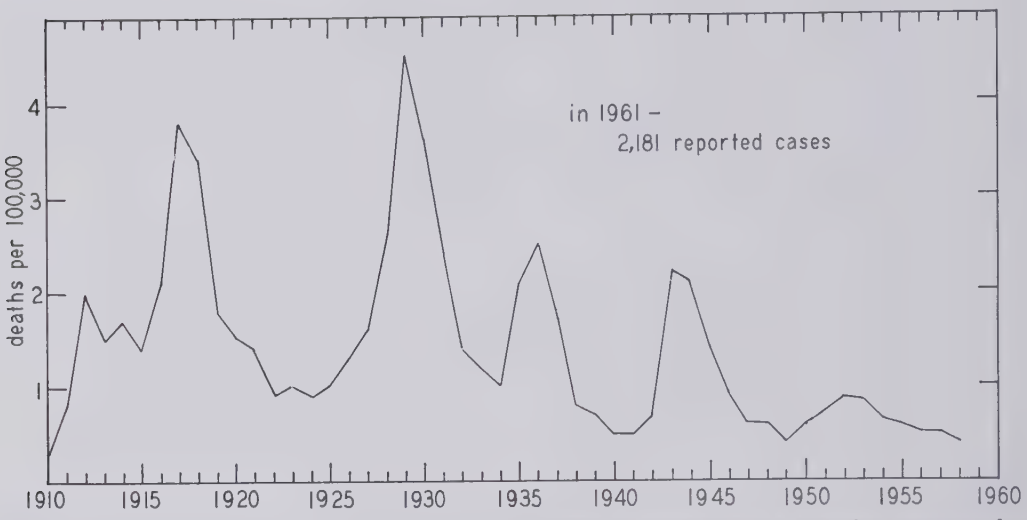


Figure 99. The prevalence of meningococcus meningitis as shown by the mortality rates in the Registration Area. Data from the Morbidity and Mortality Weekly Report, vol. 9, 1961. Communicable Disease Center, Public Health Service, U. S. Department of Health, Education and Welfare.

examination might be negative for a period, in one case as long as 4½ months, and then the same type of meningococcus would appear. In a continuous survey[46] over a 17 month period of hospital staff and employees, the peak in carrier rate of 17 per cent was reached in April and May. Of ninety carrier strains isolated, twenty did not fall into the recognized types, and seventy were typable. Of the latter group, sixteen were type I, twenty-six type II, and seven type IIα. Spouses of carriers were only occasionally carriers and then of a different type of meningococcus, suggesting a limited infectivity of the carrier strains.

The carrier rate is much higher than the incidence of the disease, and the theory that the second is a function of the first has been an intriguing one. Studies carried out during World War I suggested that there is a direct connection between the two, and that when the carrier rate exceeds 20 to 30 per cent, cases begin to appear. This relatively precise correspondence has not been substantiated, and the extensive studies of Aycock and Mueller[1] carried on during World War II showed that, while in general the prevalence of meningitis may be attributed to the prevailing type I carrier rate, variations in the incidence of disease between winter and summer, between the military and civilian populations, and between raw recruits and seasoned troops, were not associated with corresponding variations in the carrier rate.

Epidemics of meningococcus meningitis are prone to occur in military populations, and epidemic cerebrospinal fever was, with influenza, one of the most important diseases among the troops in the First World War. The influence of predisposing factors causing an increased susceptibility is especially marked under conditions of military life; fatigue, exposure to inclement weather, and similar factors very likely play a part in reducing the normal resistance of the raw recruit. This general reduced resistance, coupled with the opportunities for the dissemination of the microorganisms provided by the close communal existence in barracks, undoubtedly is an important factor in the genesis of these epidemics. Outbreaks in the civilian population differ in that children (over 3 months) and adolescents are generally attacked; susceptibility appears to be greatest in children under 10 years of age, distinctly less in adolescents, and remains at a low level thereafter.

Whether occurring in civilian or military populations, epidemics of meningo-

coccus meningitis have a number of distinctive characteristics. The relatively high carrier rate that may prevail, coupled with the low morbidity, estimated at 0.01 to 0.3 per cent of the exposed population, is an expression of a high degree of normal resistance, while the high case fatality rate indicates the serious course of the infection once established. The relative insusceptibility of the general population also results in the spotty character of the spread of the disease; some groups escape which are closely associated with the focus of infection, while in others, apparently remote, outbreaks occur. Direct transmission from case to case is not commonly observed. Usually carriers constitute the link between cases. Further, epidemics frequently consist of a series of recurring outbreaks rather than the well-marked single epidemic wave often observed to occur in other diseases. In this country during the past five decades meningococcus meningitis has occurred in epidemic waves at 6 to 12 year intervals. Since 1925 there have been three major epidemics, centering in the years 1929, 1935, and 1943. Following 1943, there was a steady decrease in the numbers of cases and deaths until 1951, when the occurrence of a slightly greater number of cases of meningococcal infection suggested the possibility that another period of increased incidence might be developing. The incidence did, in fact, rise in the United States to a peak of 5077 cases in 1953 but subsequently declined to 2181 cases in 1961.

The seasonal incidence is marked in temperate climates. Males are more frequently attacked than females, although this may be an expression of risk rather than sex differences in resistance. Racial differences in susceptibility are indicated by military experience in which the morbidity and mortality rates of the colored troops were twice those of the white.

Chemotherapy. Of the chemotherapeutic drugs presently available, the sulfonamides, especially sulfadiazine, remain the drugs of choice. The meningococcus resembles the gonococcus in its *in vitro* sensitivity to the antibiotics, but these generally diffuse poorly and do not readily enter the central nervous system; penicillin may be given by the intrathecal route but appears to offer no advantage over the sulfonamides. Under special circumstances that warrant its use, *i.e.*, the protection of recruits in the armed services, sulfadiazine is an effective prophylactic also, reducing both the carrier state and the incidence of the disease.

Bacteriological Diagnosis of Meningococcus Infection. The meningococcus is present in considerable numbers in the spinal fluid in meningitis, and the finding of the characteristic gram-negative intracellular diplococci in stained smears of the sediment from centrifuged spinal fluid is sufficient to establish a provisional diagnosis. It may be cultured by the direct inoculation of spinal fluid sediment or nasopharyngeal swabs (in the detection of carriers) on infusion base chocolate agar or blood agar. Blood culture is useful early in the disease and in those cases which do not show meningeal symptoms. In asymptomatic carriers meningococci are found in the nasopharynx, and the specimen is taken from this area by swab.[68] In any case it is important that the specimen not be allowed to cool below body temperature before inoculation of the warm medium. Like the gonococcus, the meningococcus is oxidase-positive and the oxidase test is especially useful for nasopharyngeal cultures. The isolated meningococci may be identified by fermentation tests and agglutination with polyvalent antiserum. Meningococci may be typed by agglutination, and in recent years the capsular swelling or Quellung reaction, described in the typing of pneumococci, has been used. Chicken antiserum is regarded as superior to rabbit antiserum by many workers. Typing is sometimes desirable in that a rise in the proportionate incidence of type I usually precedes an epidemic of meningococcus meningitis, and dangerous carriers may be distinguished from relatively harmless ones.[9]

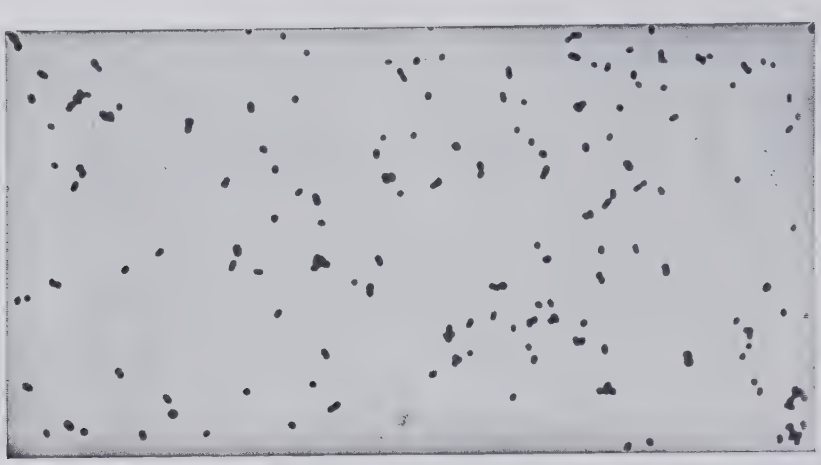


Figure 100. *Neisseria catarrhalis.* Smear from a pure culture. Note the diplococci and elongated forms which have not yet divided. Fuchsin; × 1050.

Pathogenicity for Lower Animals. The usual experimental animals are relatively resistant to the meningococcus upon intraperitoneal or intravenous inoculation. White mice are more susceptible than most other animals, and the injection of sufficient quantities of meningococci will result in death. Rabbits react similarly. Enormous numbers of bacteria must be injected and there is some question as to whether an actual infection is set up; killed meningococci are as effective as the living cells, and it is likely that the observed result is essentially a toxemia. The virulence of meningococcus for the mouse may be greatly increased by suspending the inoculum in mucin, but, as in other instances where it is necessary to use mucin, the relation of the artificial infection to the naturally occurring one is questionable.

Flexner was able to infect rhesus monkeys by the intraspinal inoculation of large amounts of meningococcus cultures, and the disease appeared to be more acute than in man. Branham and her colleagues have been able to reproduce meningococcus meningitis in rabbits and in guinea pigs by the intracisternal injection of virulent meningococci. As in man, the experimental disease is both a local meningeal involvement with purulent meningitis and general toxemia. According to Branham it is easier to produce meningitis in guinea pigs than in rabbits. Similarly, a fatal meningoencephalitis may be produced in mice by intracerebral inoculation though not by other routes. It is of interest to note that the developing chick embryo may be infected with meningococci; the 12-day embryos develop septicemia and hemorrhagic lesions simulating fulminating meningococcus septicemia in man.

Immunity. Infection with the meningococcus leads to the development of demonstrable antibodies, and patient's serum may agglutinate to a titer of 1:50. The development of agglutinins is irregular, or possibly appears so because of the antigenic instability of meningococci, and cannot be depended upon for diagnosis. Complement-fixing antibodies are produced also but too late in the disease (8 to 14 days) to be of diagnostic utility.

The injection of large number of meningococci into horses induces the formation of agglutinins, opsonins, and amboceptors. Antiserum is a reasonably effective therapeutic agent, and reduces the case fatality rate by about half, but is no longer used now that effective chemotherapeutic drugs are available.

Prophylactic vaccination with suspensions of meningococci has been attempted by a number of workers, but the procedure is difficult to evaluate. In general, there appears to be no consistent and unequivocal evidence of a significant degree of protection conferred by active immunization procedures.

OTHER GRAM-NEGATIVE DIPLOCOCCI

In addition to the gonococcus and the meningococcus, two other species of nonpigmented gram-negative diplococci are generally recognized.

Neisseria catarrhalis is found commonly in the nasopharynx of healthy individuals as well as of persons suffering from colds and other respiratory infections. The cells as a rule are somewhat smaller than those of the meningococcus. Growth occurs on ordinary nutrient agar much more readily than in the meningococcus, and the colonies are generally thicker and more opaque. Dextrose and other sugars are not fermented. Different strains vary in their pathogenicity for animals, but many strains are fully as pathogenic as meningococci for white mice. In man they appear at times to produce catarrhal inflammation, and sometimes pneumonia and meningitis have been reported. This was relatively common during the 1918 influenza epidemic in some localities.

Neisseria sicca is a small gram-negative coccus found on the mucous membranes of the respiratory tract. It grows at room temperature as well as at 37° C., forms white, firm, dry, adherent colonies, and ferments sucrose, lactose, and maltose. Although not ordinarily regarded as pathogenic, it has been found as apparently the causal agent in a case of kidney infection and has been found in the blood stream of patients ill with clinical endocarditis. This species as well as certain of the pigmented forms is, as pointed out above, found in an appreciable portion of cases of nongonococcal urethritis,[37] but its pathogenic potentialities under these circumstances are not completely clear.

The Pigmented Forms. Gram-negative diplococci which form a pale greenish yellow pigment often best observed by transmitted light may be found in the upper respiratory tract of man. Although generally considered to be nonpathogenic, they are occasionally found in disease; for example *N. perflava* and *N. flava* have been found in meningitis[69] and endocarditis.[44] These pigmented forms are differentiated from one another on the basis of fermentations.

The Anaerobic Species. The obligate anaerobic gram-negative cocci are smaller than Neisseria, 0.3 to 0.4 μ in diameter, and occur in masses and short chains as well as in pairs. These forms appear to be nonpathogenic but occur as parasites in the mouth and respiratory tract and in the intestinal and urogenital tracts as well. They are placed in a separate genus, Veillonella, and divided into two main groups on the basis of gas production in culture mediums. The gas-forming group includes *V. parvula, V. alkalescens,* and *V. discoides,* and the non-gas-forming group *V. reniformis, V. orbiculus,* and *V. vulvovaginitidis.*

REFERENCES

1. Aycock, W. L., and Mueller, J. H.: Meningococcus carrier rates and meningitis incidence. Bact. Rev., 1950, **14:**115–160.
2. Babione, R. W., and Graham, R. S.: Nongonococcal urethritis in the Navy. Amer. J. Syph., 1952, **36:**480–482.
3. Banks, H. S.: Meningococcosis. A protean disease. Lancet, 1948, **ii:**635–640, 677–681.
4. Barritt, M. M.: An improved Pappenheim stain for gonococci. Brit. Med. J., 1944, **i:**494.
5. Bittiner, J. B., and Horne, G. O.: The male gonorrhoea "carrier." Report of seven cases. Brit. J. Venereal Dis., 1955, **31:**155–159.
6. Boor, A. K.: A difference in metabolic requirements of meningococcus and gonococcus. Proc. Soc. Exptl. Biol. Med., 1942, **50:**22–25.
7. Boor, A. K., and Miller, C. P.: A carbohydrate-lipid fraction of gonococcus and meningococcus. J. Infect. Dis., 1944, **75:**47–57.
8. Branham, S. E.: The meningococcus. Bact. Rev., 1940, **4:**59–96.
9. Branham, S. E.: Value of typing meningococci. Amer. J. Pub. Health, 1945, **35:**233–238.
10. Branham, S. E.: Serological relationships among meningococci. Bact. Rev., 1953, **17:**175–188.
11. Brown, W. J.: The status of gonorrhoea in the USA and current problems in its control. Bull. W. H. O., 1961, **24:**386–394.
12. Campbell, E. P.: Meningococcemia. Amer. J. Med. Sci., 1943, **206:**566–576.
13. Carpenter, C. M.: Gonococcal resistance to penicillin in the light of recent literature. Bull. W. H. O., 1961, **24:**321–326.
14. Chanarin, I.: An investigation of *Neisseria gonorrhoeae* by a red cell sensitization technique. J. Hyg., 1954, **52:**425–443.
15. Cohn, A., Steer, A., and Adler, E. L.: Gonococ-

cal vaginitis—a preliminary report on one year's work. Venereal Dis. Inform., 1940, **21**:208–220.
16. Cradock-Watson, J. E., Shooter, R. A., and Nicol, C. S.: Sensitivity of strains of gonococci to penicillin, sulphathiazole and streptomycin. Brit. Med. J., 1958, **i**:1091–1092.
17. Daniels, W. B.: Meningococcic bacteremia. Arch. Intern. Med., 1948, **81**:145–161.
18. Davidson, H. H., Hill, J. H., and Eastman, N. J.: Penicillin in the prophylaxis of ophthalmia neonatorum. J. Amer. Med. Assn., 1951, **145**: 1052–1055.
19. Deacon, W. E.: Fluorescent antibody methods for *Neisseria gonorrhoeae* identification. Bull. W.H.O., 1961, **24**:349–355.
20. Del Love, B., Jr., and Finland, M.: Susceptibility of *Neisseria gonorrhoeae* to eleven antibiotics and sulfadiazine. Arch. Intern. Med., 1955, **95**:66–73.
21. Drell, M. J., Miller, C. P., Bohnhoff, M., and Moeller, V.: Experimental gonococcal infection of the rabbit's eye. II. Course of the disease and its pathology. J. Infect. Dis., 1945, **77**:201–215.
22. Durel, P., Roiron, V., and Delouche, L.: Problèmes posés par l'emploi de la streptomycine dans le traitment de la gonococcie. Bull. W. H. O., 1961, **24**:343–348.
23. Fairbrother, R. W.: Meningococcal meningitis with particular reference to epidemiology and pathogenesis. J. Clin. Path., 1947, **1**:10–18.
24. Freyche, M. J.: World distribution and trend of cerebrospinal meningitis since 1939. Epidemiol. Vital Stat. Rept. W. H. O., 1951, **4**: 310–351.
25. Freeble, C. R., Jr., and Donohue, J. F.: Syphilis and gonorrhea as causes of blindness. A study of persons declared legally blind in Ohio. J. Amer. Med. Assn., 1951, **146**:1500–1506.
26. Gisslen, L., Hellgren, L., and Starck, V.: Incidence, age distribution and complications of gonorrhoea in Sweden. Bull. W. H. O., 1961, **24**:367–372.
27. Gould, R. G., Kane, L. W., and Mueller, J. H.: On the growth requirements of *Neisseria gonorrhoeae*. J. Bact., 1944, **47**:287–292.
28. Gover, M., and Jackson, G.: Cerebrospinal meningitis. A chronological record of reported cases and deaths. Pub. Health Repts., 1946, **61**:433–450.
29. Griffin, P. J., and Racker, E.: The carbon dioxide requirement of *Neisseria gonorrhoeae*. J. Bact., 1956, **71**:717–721.
30. Griffin, P. J., and Rieder, S. V.: A study on the growth requirements of *Neisseria gonorrhoeae* and its clinical application. Yale J. Biol. Med., 1957, **29**:613–621.
31. Grossowicz, N.: Growth requirements and metabolism of *Neisseria intracellularis*. J. Bact., 1945, **50**:109–115.
32. Guthe, T.: Failure to control gonorrhoea. Bull. W.H.O., 1961, **24**:297–306.
33. Harkness, A. H.: The pathology of gonorrhoea. Brit. J. Venereal Dis., 1948, **24**:137–147.
34. Harkness, A. H.: Non-gonococcal urethritis. Pub. Health, 1954, **68**:10.
35. Hedrich, A. W.: Recent trends in meningococcal disease. Pub. Health Repts., 1952, **67**:411–420.
36. Herbut, P. A., and Manges, W. E.: Fulminating meningococcic infection. (The Waterhouse-Friderichsen syndrome). Arch. Path., 1943, **36**:413–422.
37. Johnston, J.: Nongonococcal Neisserian strains isolated from the genitourinary tract. Amer. J. Syph., 1951, **35**:79–82.
38. Kabat, E. A., Kaiser, H., and Sikorski, H.: Preparation of the type-specific polysaccharide of the type I meningococcus and a study of its effectiveness as an antigen in human beings. J. Exptl. Med., 1944, **80**:299–307.
39. King, A. J., and Nicol, C. S.: The problem of gonorrhoea in England and Wales. Bull. W. H. O., 1961, **24**:373–384.
40. Lankford, C. E., Scott, V., Cox, M. F., and Cooke, W. R.: Some aspects of nutritional variation of the gonococcus. J. Bact., 1943, **45**:321–326.
41. Macrae, J.: Meningococcal meningitis. Postgraduate Med. J., 1955, **31**:92–96.
42. Martland, H. S.: Fulminating meningococcic infection with bilateral massive adrenal hemorrhage (the Waterhouse-Friderichsen syndrome). With special reference to the pathology, the medicolegal aspects and the incidence in adults. Arch. Path., 1944, **37**:147–158.
43. Mathieu, P. L., Jr.: Comparison study: Silver nitrate and oxytetracycline in newborn eyes. A comparison of the incidence of conjunctivitis following instillation of silver nitrate or oxytetracycline in the eyes of newborn infants. J. Dis. Children, 1958, **95**:609–611.
44. Matlage, W. T., Harrison, P. E., and Greene, J. A.: *Neisseria flava* endocarditis: With report of a case. Ann. Intern. Med., 1950, **33**:1494-1498.
45. Mead, R. K., Moon, N. F., and Bean, L. L.: Penicillin-resistant gonorrheal urethritis in male personnel. U. S. Armed Forces Med. J., 1960, **11**:1117–1123.
46. Miller, C. P., Baedenkopf, W., Peck, D., and Robbins, M. S.: A survey of chronic meningococcus carriers in a semi-permanent population. J. Infect. Dis., 1944, **74**:212–224.
47. Miller, C. P., Bohnhoff, M., and Moeller, V.: Experimental gonococcal infection of the rabbit's eye. III. Treatment with prophylactic and therapeutic agents. J. Infect. Dis., 1945, **77**: 216–223.
48. Miller, C. P., Drell, M. J., Moeller, V., and Bohnhoff, M.: Experimental gonococcal infection of the rabbit's eye. I. Method of production. J. Infect. Dis., 1945, **77**:193–200.
49. Milner, K. C., and Shaffer, M. F.: Type-specific capsular swelling of meningococci by chicken antiserum. Proc. Soc. Exptl. Biol. Med., 1946, **62**:48–49.
50. Morton, H. E., and Shoemaker, J.: The identification of *Neisseria gonorrhoeae* by means of bacterial variation and the detection of small colony forms in clinical material. J. Bact., 1945, **50**:585–587.
51. Mullaney, P. J.: A simple fermentation medium for *Neisseria gonorrhoeae*. J. Path. Bact., 1956, **71**:516–517.
52. Murray, E. G. D.: The meningococcus. Med. Res. Council (Great Britain) Spec. Rept. Ser. No. 124, 1929.
53. Nelson, J., and Goldstein, N.: Nature of Waterhouse-Friderichsen syndrome. Report of a case with successful treatment with cortisone. J. Amer. Med. Assn., 1951, **146**:1193–1197.
54. Nicol, C. S.: Non-specific urethritis. Pub. Health, 1954, **68**:10–11.
55. Parrino, P. S.: Nongonococcic urethritis in the male. U. S. Armed Forces Med. J., 1954, **5**: 1249–1266.
56. Parrino, P. S., O'Shaughnessy, E. J., and White,

J. D.: Standardization of diagnostic methods for gonococcal infections. Amer. J. Pub. Health, 1955, **45:**457–463.
57. Phair, J. J., and Schoenbach, E. B.: The transmission and control of meningococcal infections. Amer. J. Med. Sci., 1945, **209:**69–74.
58. Phair, J. J., Schoenbach, E. B., and Root, C. M.: Meningococcal carrier studies. Amer. J. Pub. Health, 1944, **34:**148–154.
59. Public Health Service: Gonococcus: Procedures for isolation and identification. U. S. Department of Health, Education and Welfare. Public Health Service Publication No. 499, 1957.
60. Reyn, A.: Serological studies on gonococci. I. Technique. Gono-reaction of "normal" rabbits. Serological relation between gonococci and Pasteurellae. Acta Path. Microbiol. Scand., 1949, **26:**51–70.
61. Reyn, A.: Serological studies on gonococci. II. Cross-absorption experiments and factor serum determinations. Acta Path. Microbiol., Scand., 1949, **26:**234–251.
62. Reyn, A.: Serological studies on gonococci. III. Thermostability and biochemical nature of the gonococcus antigens. Durability of the factor sera. Discussion. Acta Path. Microbiol. Scand., 1949, **26:**252–268.
63. Reyn, A., and Bentzon, M. W.: Possible effects of antibiotic treatment on the sensitivity and growth requirements of *Neisseria gonorrhoeae*. Bull. W.H.O., 1961, **24:**333–342.
64. Roiron, V., Rasetti-Nicod, G., and Durel, P.: Étude de la sensibilité actuelle de gonocoque aux principaux antibiotiques. Ann. Inst. Pasteur, 1961, **100:**445–462.
65. Schamberg, I. L.: Unknowns and enigmas in gonorrhea. Pub. Health Repts., 1956, **71:**537–544.
66. Scherp, H. W.: Neisseria and Neisserial infections. Ann. Rev. Microbiol., 1955, **9:**319–334.
67. Scherp, H. W., and Fitting, C.: The growth of *Neisseria meningitidis* in simple chemically defined media. J. Bact., 1949, **58:**1–9.
68. Schoenbach, E. B., and Phair, J. J.: Appraisal of the techniques employed for the detection of subclinical (inapparent) meningococcal infections. Amer. J. Hyg., 1948, **47:**271–281.
69. Sophian, L. H.: A case of acute meningitis caused by *Neisseria perflava*. Amer. J. Med. Sci., 1944, **207:**376–378.
70. Stuart, R. D.: Transport medium for specimens in public health bacteriology. Pub. Health Repts., 1959, **74:**431–438.
71. Symposium: The clinical and administrative aspects of vulvo-vaginitis. Med. Officer, 1938, **59:**191–195; 203–206.
72. Tauber, H., and Garson, W.: Isolation of lipopolysaccharide endotoxin. J. Biol. Chem., 1959, **234:**1391–1393.
73. Tauber, H., and Russell, H.: Amino compounds in lipopolysaccharides. J. Biol. Chem., 1960, **235:**961–964.
74. Tauber, H., and Russell, H.: Biochemistry of *Neisseria gonorrhoea* endotoxin. Bull. W.H.O., 1961, **24:**385–386.
75. Thayer, J. D., et al.: Surveillance studies of *Neisseria gonorrhoeae* sensitivity to penicillin and nine other antibiotics. Bull. W.H.O., 1961, **24:**327–332.
76. Thayer, J. D., Perry, M. I., Magnuson, H. J., and Garson, W.: Failure of penicillin to kill phagocytosed *Neisseria gonorrhoeae* in tissue culture. Antibiot. Chemotherap., 1957, **7:**311–314.
77. Thayer, J. D., Schubert, J. H., and Bucca, M. A.: The evaluation of culture mediums for the routine isolation of the gonococcus. Venereal Dis. Inform., 1947, **28:**37–40.
78. Watson, R. G., Marinetti, G. V., and Scherp, H. W.: The specific hapten of group C (group II_α) meningococcus. II. Chemical nature. J. Immunol., 1958, **81:**337–344.
79. Watson, R. G., and Scherp, H. W.: The specific hapten of group C (group II_α) meningococcus. I. Preparation and immunological behavior. J. Immunol., 1958, **81:**331–336.
80. Wilkinson, A. E.: Occurrence of Neisseria other than the gonococcus in the genital tract. Brit. J. Venereal Dis., 1952, **28:**24–27.
81. Willcox, R. R.: Treatment problems of gonorrhoea. Bull. W.H.O., 1961, **24:**307–320.
82. Willcox, R. R.: Epidemiological aspects of gonoccal infections. Bull. W.H.O., 1961, **24:**357–366.
83. Wilson, J. F.: A serological study of *Neisseria gonorrhoeae*. J. Path. Bact., 1954, **68:**495–510.
84. Wilson, J. F.: A serological study of the meningococcus. J. Path. Bact., 1956, **72:**111–119.
85. World Health Organization: Cerebro-spinal meningitis. Its recent trend. Epidemiol. Vital Stat. Rept. W.H.O., 1952, **5:**617–623.

Chapter 20

THE ENTERIC BACILLI

The Coliform Bacteria, Friedländer's Bacillus (Pneumobacillus), and Proteus

THE GRAM-NEGATIVE, non-spore-forming bacilli make up a large group of bacteria which includes intestinal commensals such as the colon bacilli and Proteus; the enteric pathogens such as the typhoid, paratyphoid, and dysentery bacilli; certain saprophytic forms and plant pathogens; and, as more distant relatives, the hemophilic bacteria (Hemophilus); the so-called hemorrhagic septicemia group (Pasteurella); and the causative microorganisms of undulant fever (Brucella).

The largest of these groups, and the subject of this and immediately following chapters, is that of the enteric bacilli, or Enterobacteraceae. These bacteria are found in the intestinal tract of man and other warm-blooded animals, as commensals of limited pathogenic potentialities associated with diarrheal disease, or, and much less commonly, in infections of the tissues. There are other gram-negative bacilli which are more or less related to the enteric bacilli, but are differentiable from them.

Those grouped in the genus Serratia[20] are formally a part of the Enterobacteraceae by virtue of cultural similarities, but are for the most part free-living saprophytes. Occasionally they are found in association with, and possibly causal relation to, pathological processes.[28] The most familiar of these is the red pigmented *Serratia marcescens*, or *Chromobacterium prodigiosum*. The pigment is not apparent in cultures incubated at 37° C., but the colonies are a bright pink-red at lower temperatures. A majority of the Serratia are not pigmented, and in general these bacteria tend to be somewhat less active biochemically than many of the enteric bacilli.

The gram-negative bacilli of the Aeromonas group[23, 24] superficially resemble the enteric bacilli, and in fact certain strains, originally designated C27 paracolon bacilli, contain antigens also found in the Sonne dysentery bacilli. They also resemble Proteus in having polar flagella, and certain of them, like some strains of Proteus, are pathogenic for cold-blooded animals. A considerable number of species have been described from time to time, but these can be put into one or another of three species, *viz.*, *Aeromonas hydrophila*, which produces red-leg disease of frogs; *A. salmonicida*, responsible for disease of trout and other fish; and *A. shigelloides*, antigenically related to dysentery bacilli. Aeromonas has rarely been found in association with disease of warm-blooded animals, including man, and is apparently nonpathogenic for them.

Still other bacteria are closely related

to the enteric bacilli, in fact sometimes cannot be distinguished from them with certainty, but are set apart by differences in habitat. Such are Friedländer's bacillus, a common inhabitant of the upper respiratory tract and the causative agent of a small proportion of pneumonias, and the plant pathogens of the genus Erwinia which produce soft rots of vegetables. Similarly, the members of the genera Proteus and Pseudomonas occur as free-living saprophytes as well as intestinal commensals of occasional pathological significance.

CLASSIFICATION OF ENTERIC BACILLI

The differentiation and characterization of the enteric bacilli, or Enterobacteriaceae, is based upon a variety of biochemical and cultural reactions and upon antigenic structure; these bacteria are better known by these conventional criteria than any other group of microorganisms. When the more important of them, the typhoid and paratyphoid bacilli, the colon bacilli, and the dysentery bacilli, were first described and studied, it was not difficult to characterize them with reasonable precision, and they appeared to fall into genera and species in the limited sense in which these concepts seem to be applicable to microorganisms.

With increasing knowledge, it has become obvious that the enteric bacilli make up a continuous series of forms, showing almost every conceivable combination of differential characteristics. Combinations which have not yet been found in nature have, in some instances, been created in the laboratory by recombination and transduction, with demonstrable fertility groups crossing "genus" lines (Chap. 7). Sharp distinctions are no longer possible, with different kinds of enteric bacilli merging imperceptibly into one another.

The inadequacy of the usual differential criteria as a basis for a formal classification or taxonomy, as distinct from a differential key, is clearly evident in this group of bacteria. In fact, it is now becoming generally realized that subdivision of the family Enterobacteriaceae into the conventional tribes, species, and genera cannot be made on other than a genetic basis, which is as yet lacking, and that the species concept in the Linnaean sense is not applicable. At best, the enteric bacilli can be separated into groups, subgroups, and serotypes for purposes of practical expediency[35]

Groups and Subgroups of Enterobacteriaceae[7, 21]

Salmonella-Arizona	Salmonella (typhoid and paratyphoid bacilli)
	Arizona (Salmonella-like paracolon bacilli)
Citrobacter, Bethesda-Ballerup	Citrobacter (intermediate type of colon bacilli)
	Bethesda-Ballerup (slow lactose-fermenting Citrobacter or Salmonella-like paracolon bacilli)
Shigella-Escherichia	Shigella (the dysentery bacilli)
	Escherichia (the classic colon bacillus and the alkalescens and dispar dysentery bacilli)
Klebsiella-Aerobacter-Serratia	Klebsiella (Friedländer's pneumobacillus)
	Aerobacter (the classic aerogenes bacilli), Hafnia
	Serratia
Proteus-Providence	Proteus
	Providence group of paracolon bacilli

Biochemical Reactions of Enteric Bacilli

ENTERIC BACILLI		BIOCHEMICAL REACTIONS																						
Group	Species	Butt, Russell's Slant		Dextrose	Lactose	Sucrose	Mannitol	Dulcitol	Sorbitol	Maltose	Rhamnose	Raffinose	Arabinose	Trehalose	Inositol	Xylose	Salicin	Milk	H_2S	Indol	Gelatin	d-Tartrate	l-Tartrate	m-Tartrate
Coliform Bacilli	*A. aerogenes*	G	a	G	G	G	G	G —	G	G	G	G	G	G	G	G	G	ac	—	—	—			
	E. coli var. *communior*	G	a	G	G	G	G	G —	G	G	G	G	G	G	—	G	—	ac	—	+	—			
	E. coli var. *communis*	G	a	G	G	—	G	G —	G	G	G	G —	G	G	—	G —	G	ac	—	+ —	—			
	A. cloacae	G	ak	G	G	G	G	G —	G	G	G	G	G	G	a	G	G	ac	—	+ —	+			
Dysentery Bacilli	*Sh. sonnei*	a	— or k	a	a (l)	a (l)	a	—	—	a (l)	a	a	a	a	—	— a	—	a(l) ac	—	—	—			
	Sh. shigae	a	— or k	a	—	—	—	—	—	—	—	a	—	a —	—	—	—	ak	—	—	—			
	Sh. flexneri	a	— or k	a	—	a —	a	— a	a —	a —	— a	a —	a —	a	a —	—	a —	ak	—	+ —	—			
	Sh. ambigua (Schmitz)	a	— or k	a	—	—	—	—	— a	—	a		—			—		ak	—	+	—			
	Sh. alkalescens	a	— or k	a	—	a —	a	a	a	a	a					a	—	k	—	+	—			
	E. dispar var. *ceylonensis*	a	— or k	a	a (l)	a (l)	a	a	a	a (l)	a	a	a		—	a	—	a(l) ac	—	+	—			
	E. dispar var. *madampensis*	a	— or k	a	a (l)	a (l)	a	—	a (l)	a (l)	a		a		—	a	—	a(l) ac	—	+	—			
Salmonella	*Sal. typhi*	a	ak	a	—	—	a	a(l)	a	a	—	a	— a	a	—	a	—	ak	+	—	—	+	—	—
	Sal. gallinarium	a	ak	a	—	—	a	a	—	a	a	—	a	a	—	a(l)	—	—	— +	—	—	+	+	— +
	Sal. pullorum	G	ak	G	—	—	G	—	—	—	G	—	G	G	—	G	—	ak	+	—	—	—	— +	—
	Sal. paratyphi A	G	k	G	—	—	G	G	G	G	G	—	G	G	—	—	—	ak	—	—	—	—	—	—
	Sal. paratyphi B	G	k	G	—	—	G	G	G	G	G —	— G	G	G	a —	G	—	ak	++	—	—	+ —	+	+ —
	Sal. typhi-murium	G	ak	G	—	—	G	G	G	G	— G	—	G	G —	G —	G	—	ak	++	—	—	+ —	+	+ —
	Sal. paratyphi C (Hirschfeld)	G	ak	G	—	—	G	G	G	G	G		G	G	—	G	—	ak	++	—	—	+	+(l)	—
	Sal. cholerae-suis (American)	G	ak	G	—	—	G	— G	G	G	G	—	—	—	—	G	—	ak	— +	—	—	+	—	— +
	Sal. cholerae-suis var. *kunzendorf*	G	ak	G	—	—	G	— G	G	G	G	—	—	—	—	G	—	ak	++	—	—	+ —	— +	— +
	Sal. typhi-suis (Glässer-voldagsen)	G	ak	a	—	—	— a(l)	a —	a —	a —	a —		— a	a	—	a	—	—	—	—	—	—	—	—
Others	*Alcaligenes fecalis*	—	k	—	—	—	—	—	—	—	—	—	—	—	—	—	—	k	+	—	—	—	—	—
	Ps. aeruginosa (*pyocyanea*)	—	k	—	—	—	—	—	—	—	—	—	—	—	—	—	—	k	+	—	—	—	—	—
	Proteus vulgaris	G	k	G	—	G	—			— G						G —		kd	++	+	+			
	Proteus mirabilis	G	k	G	—	G(l) —	—			—								kd	++	—	+			
	Proteus morgani	G	k	G	—	—	—	—	—	—		—	—		—	— G	—	k	—	+	—			
	Proteus rettgeri	G	k	a	—	a(l)	a			—							a —	kd	—	+	—			

G—acid and gas; a—acid; G or a—most strains ferment; $\bar{G}$ or $\bar{a}$—most strains do not ferment. (l)—late; k—alkaline; ak—acid to alkaline; c—curd; d—digestion; + —utilization of tartrate, formation of H_2S or indol, or liquefaction of gelatin; — —no action.

and the construction of an elaborate and ambitious taxonomy of these microorganisms, and by inference possibly others also, is futile.

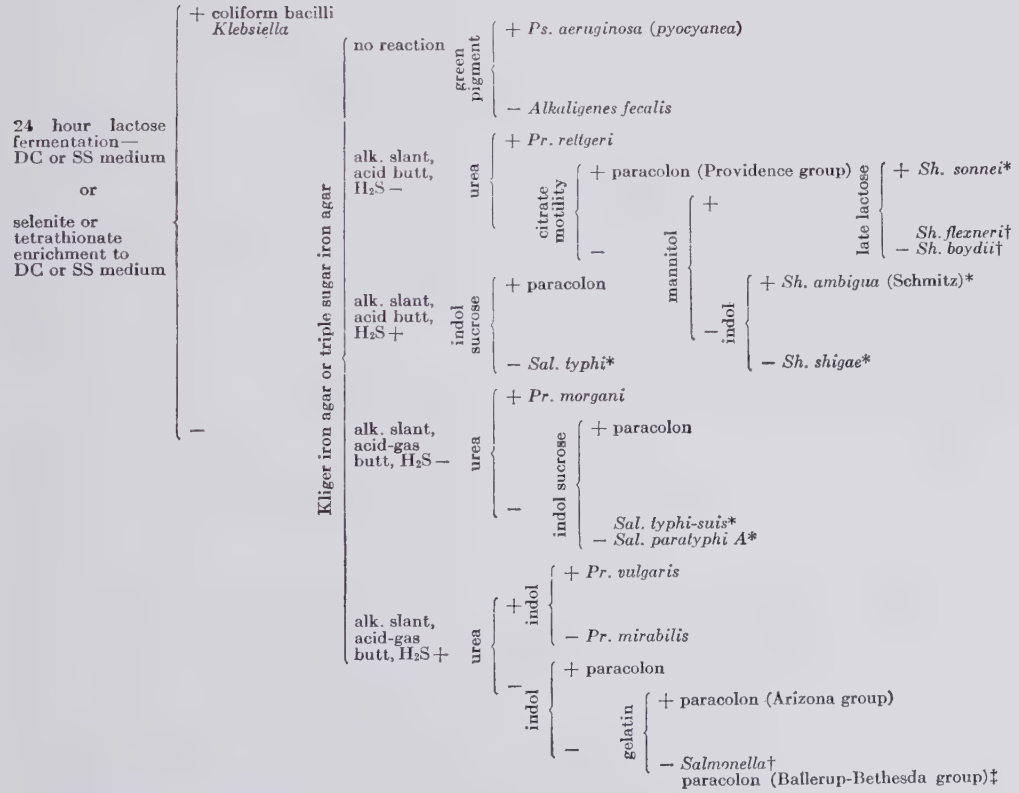
Nomenclature is another and distinct matter and some workable system is essential.[8] Certain names, originally introduced as generic names, including Salmonella, Shigella, Escherichia, Klebsiella, and Serratia, have gained general acceptance, while others, especially certain species designations, have not. A practical basis of a nomenclature is usage, and therefore familiarity, rather than the priority demanded by the purist, for the latter has often resulted in the creation of new and unfamiliar genera and species which correspond neither with common international practice nor the great bulk of research literature, past and current. Here nomenclature in the controversial area of the enteric bacilli will be based on usage, and names are regarded as no more than useful handles without phylogenetic and taxonomic significance.

Biochemical Differentiation.[11, 17] A useful primary differentiation can be made on the basis of the lactose fermentation which is roughly correlated with pathogenicity; the coliform bacteria ferment this sugar rapidly with the formation of acid and gas in 24 hours, while the bacteria of the other groups, essentially pathogens (Shigella, Salmonella), do not ferment it. The distinction is not absolute, of course, since both the paracolon bacilli and certain of the dysentery bacilli are slow lactose fermenters, but is sufficiently marked to have considerable practical value.

Similarly, the dysentery bacilli, or Shigellas, divide into two groups on the basis of the fermentation of mannitol

Differentiation of the Pathogenic Enteric Bacilli

(Arranged by R. S. Benham)



* immunologically homogeneous
† subdivided into immunological species or types
‡ ordinarily show late lactose and sucrose fermentation

and are anaerogenic, that is to say, do not produce gas from fermented carbohydrates. While the Salmonella group in general produces gaseous fermentations, the typhoid bacillus and *Salmonella gallinarum* are typically anaerogenic, and anaerogenic strains of other Salmonellas are occasionally met with. The general biochemical groups are indicated in the accompanying table of biochemical reactions.

The forms occurring in specimens from pathological processes in man are separable by systemic application of these biochemical characteristics, both as in the composition of mediums made selective by the inclusion of bile salts and differential by sugars and an indicator, and by specific biochemical tests. The practical application of these differential methods is illustrated in the accompanying scheme.

THE COLIFORM BACILLI

The coliform bacilli were described by Escherich in 1886 as *Bacterium coli commune* and *Bact. lactis aerogenes.* It was soon evident that the former occurs as two fermentative types, *Bact. coli communior* which ferments sucrose, and *Bact. coli communis* (or *commune*) which does not. Still another type, *Bact. coli anaerogenes,* ferments sugars without the production of gas. The type characterized by a ready mutation with respect to β-galactosidase, *Bact. coli mutabile,* has been discussed elsewhere (Chap. 7). The microorganism described by Friedländer in 1883 as a causative agent of pneumonia, and known as Friedländer's bacillus or pneumobacillus, is closely related to *Bact. lactis aerogenes.* In addition to these, coliform bacilli characterized by a delayed or late (5 to 8 days) fermentation of lactose long occupied an anomalous position and were eventually called paracolon bacilli.

These forms are widely distributed and ubiquitous in the sense that they are universally found in the intestinal tract of man and many of the higher animals. Some varieties appear to occur free in nature, but it is probable that these represent contamination, although possibly distant, and these bacteria are primarily parasites of animals.

Morphology and Staining. The coliform bacilli exhibit considerable variation in their morphology. The usual dimensions observed in stained preparations from cultures upon nutrient agar or gelatin range from 2 μ to 4 μ in length and from 0.4 μ to 0.7 μ in breadth. Very short, oval and coccus-like forms are often found and usually predominate when the bacillus is observed directly in the normal animal. The bacilli are occasionally observed in pairs or short chains. Some varieties are encapsulated, particularly those found in pathological conditions. Motility is variable, although the most typical strains are motile by peritrichous flagella. Spores are not formed.

Colonies upon nutrient gelatin are more consistent and characteristic in appearance than those upon agar mediums. They are opaque to partially translucent, smooth, moist, and homogenous in consistency, with entire to undulating edge, and exhibit the maple-leaf appearance common to many of the enteric bacteria. Colonial morphology is somewhat variable upon nutrient agar. Typical colonies are opaque and grayish white, and there may be a tendency to become a light yellowish brown upon continued incubation. Pigmented varieties are occasionally observed. In certain differential mediums the colonies of coliform bacilli may assume other characteristics typical under the special circumstances. Upon Endo's medium, for example, the colonies are, of course, red, but in addition take on a curious metallic sheen that is highly characteristic when viewed by reflected light. Some varieties are β-hemolytic on blood agar; such hemolytic

strains occur with much greater frequency in pathological processes than in the normal intestine.

Coliform bacilli stain readily with the ordinary aniline dyes and are gram-negative. Flagella may be demonstrated by special staining methods.

Physiology. The coliform bacilli are facultative anaerobes, growing equally well under aerobic or completely anaerobic conditions. They grow luxuriantly upon the ordinary nutrient mediums and may be cultivated in synthetic solutions containing an ammonium salt and an organic source of carbon such as glucose. Growth occurs over a temperature range of 10° to 46° C., there is good growth from 20° to 40° C., and the optimum is 37° C.

Milk is curdled with an acid reaction usually within 48 hours. Gelatin liquefaction, hydrogen sulfide production, and the formation of indol vary from one kind of coliform bacilli to another and have differential value.

Various sugars are actively fermented with the production of acid and gas. The ability to ferment a wide variety of sugars and other polyhydric alcohols is variable, and some of the fermentations are used for differentiating the various kinds of coliform bacilli. The greater part of the acid produced is lactic acid; smaller quantities of formic and acetic acids are formed, together with ethyl alcohol. Succinic acid is also found in variable but small amounts.

The coliform bacilli are ordinarily resistant to deleterious influences, being, as a rule, neither as resistant as staphylococci nor as susceptible as the more delicate bacteria. Most strains are killed by exposure to 60° C. for 30 minutes, but occasionally more resistant varieties are encountered. In common with the other gram-negative bacteria, they are considerably less susceptible to the bacteriostatic action of dyes than are the gram-positive microorganisms, and selective mediums containing dyes are commonly used in the primary isolation of the enteric bacteria. The ability of the coliform bacilli to grow in the presence of bile is likewise made use of in selective mediums (MacConkey's broth) in the bacteriological examination of water.

Variation. The existence of two colony types of coliform bacilli has long been known, the one the flat, maple-leaf form, and the other a smaller, raised, round, moist type. The colon bacillus dissociates into rough and smooth colonial types, the S form giving rise to smooth, round, domed, shiny, translucent colonies, while the R colony type is characterized by an irregular, dull surface, jagged outline and opacity. It has been suggested that the R form is the more virulent.

Practically all kinds of microbial variation are not only known to occur among the coliform bacilli, but these have been by far the most commonly used bacteria for such experimental purposes. The K-12 strain, for example, was used in the first recombination experiments, the *mutabile* strains provided one of the first and most thoroughly studied examples of mutation, and enzyme adaptation studies have been carried out to a considerable extent with these bacteria.

Toxins. As in the case of many other gram-negative bacteria, the cell substance is toxic to experimental animals upon parenteral inoculation. This endotoxin, presumably occurring as a lipid-polysaccharide-polypeptide complex, is separable as a toxic lipopolysaccharide, and is generally considered to be the prototype of bacterial endotoxins (Chap. 9). A number of strains are hemolytic on blood agar culture, as noted above, and may also produce filterable hemolysins; these have not been studied at any length.[1]

PHYSIOLOGICAL DIFFERENTIATION OF COLIFORM BACILLI

The coliform bacilli are separated into three groups on the basis of four biochemical reactions, and one of the groups further split using motility and gelatin liquefaction as criteria. The four biochemical reactions are the formation of indol from tryptophan, the methyl red

test, the Voges-Proskauer (V-P) reaction,[49] and the ability to utilize citrate as a sole source of carbon.

The methyl red test is a determination of the pH of a dextrose broth culture after 2 to 4 days' incubation. The indicator is added to the incubated culture, and the test is said to be positive when the accumulated acidity is sufficient to turn the indicator red, and negative when the indicator remains yellow.

The Voges-Proskauer reaction is a qualitative test for the presence of acetyl-methylcarbinol among the later end products of glucose fermentation (Chap. 5). After 2 to 4 days' growth in glucose-peptone water medium, 5 ml. of a 10 per cent solution of KOH is added. On standing in the presence of alkali, the acetyl-methylcarbinol is oxidized to diacetyl which in turn reacts with some constituent of the peptone to give a pink color. When acetyl-methylcarbinol is produced, the bacterial strain is said to be V-P positive, and when it is not, V-P negative.

These four tests are fixed in order by the mnemonic "Imvic" or "IMViC" and are known as the imvic reactions. While there are sixteen possible combinations of these reactions and, in fact, coliform strains giving each of these have been found, three groups are differentiated as indicated in the accompanying table. The classic colon bacillus, *Escherichia* (*Bacterium*) *coli,* is + + − −, and the Aerobacter (Cloaca)-Klebsiella group is − − + +. Only one intermediate group, − + − +, is recognized; the bacteria of this group have been called *E. freundii* and are now designated *Citrobacter freundii.* Strains showing this combination of the imvic reactions are also called intermediate strains, or *E. coli intermedium,* which has no formal status. Other combinations have no formal name and are designated "irregular." The relative distribution of these coliform types is shown in the accompanying table.

A number of other tests of biochemical activity are valuable in differentiating the coliforms from one another and from other enteric bacilli. These include the ability to grow in the presence of 1:13,000 KCN, the deamination of phenylalanine to phenylpyruvic acid, the utilization of sodium malonate, and decarboxylase activity with respect to arginine, lysine and ornithine.[19, 39] The usefulness of such tests varies from one group to another; for example, the utilization of malonate is useful in differentiation within the Klebsiella-Aerobacter-Serratia group, and and the decarboxylase reactions in the Aerobacter, Proteus, and Providence groups as well as in enteric bacilli other than coliforms. The utilization of the isomers of tartaric acid, used in the characterization of Salmonella species prior to the extensive development of serological typing, has been revived,[36] and applied more broadly. The physiological character of the coliforms has been compiled and summarized.[42]

The Aerobacter, or Cloaca, group is, as indicated above, broken down into two parts, Aerobacter (Cloaca) and Klebsiella. The former is widely known as *Aerobacter aerogenes,* corresponding to Escherich's *Bact. lactis aerogenes.* By definition, *A. aerogenes* is motile and fails to liquefy gelatin, while Klebsiella is nonmotile and liquefies gelatin slowly. The combination of properties represented by *A. aerogenes* is, in fact, extremely rare, and most strains which are motile also liquefy gelatin and are, again by definition, *A.* (*Cloaca*) *cloacae,* in the older literature *Bact. cloacae.* Thus the name *A. aerogenes,* widely used for coliform strains in genetic and physiological studies and in water bacteriology (Chap. 11), tends to drop out of existence. It has been suggested[30] that this situation be resolved by creation of a genus En-

Percentage Distribution of Coliform Types*

SOURCE	COLI	AER.	INT.	IRR.	TOTAL
Milk	28.8	49.5	20.3	1.4	2224
Water	51.6	28.6	18.5	1.3	9496
Soil	23.8	54.3	18.8	3.1	1330
Grains	17.9	73.8	7.3	1.0	587
Feces	87.9	5.2	6.8	0.1	3974

* As compiled from various authors. In many instances the strains studied were not isolated at random; the percentages are, therefore, weighted, and great significance cannot be attached to them.

terobacter to include Aerobacter as two differentiable kinds, cloaceae and aerogenes, leaving Klebsiella to include the nonmotile forms.

In summary the coliform bacilli are separated into the following kinds: *Escherichia coli, Citrobacter* (*Escherichia*) *freundii,* the rare form *Aerobacter aerogenes, Aerobacter* (*Cloaca*) *cloacae,* and *Klebsiella pneumoniae,* the last Friedländer's bacillus.

THE IMMUNOLOGICAL RELATIONSHIPS OF THE COLIFORM BACILLI

The antigenic structure of the colon bacilli has been elucidated in great detail, and the coliforms may be identified as serotypes with a high degree of precision.[18, 27, 32] There appear to be three kinds of antigens present, *viz.:*

(1) Heat-stable O antigens, of which more than 100 have been found. Of these, twenty-five occur with sufficient frequency for diagnostic use with most strains of coliform bacteria.

(2) Somatic surface antigens, designated as "envelope" antigens or K antigens. These function as "blocking antigens" in that their presence interferes with agglutination with O antiserums. Three kinds of K antigens have been described:

(a) Those which are designated L antigens are thermolabile, and the O agglutinability of bacterial suspensions is restored by boiling. L antiserums may be prepared by absorption of LO serums with the homologous O antigen, *viz.,* boiled bacteria. The colonies of strains containing L antigens are somewhat more opaque than those which do not contain them. About twenty-four L antigens have been described.

(b) The component of the K antigen designated the A antigen is present in encapsulated coliform bacilli, is a specific polysaccharide, and bacteria containing it give a Quellung reaction in antiserum. It differs from the L antigen in that it is thermostable. So-called N variants lacking the antigen are found in translucent areas at the edge of the large, dense, and relatively opaque colony. About twenty A antigens have been found.

(c) An antigenic component of the K antigen complex, designated the B antigen, is thermolabile but differs from the L antigen in that the heated antigen can absorb antibody though heated suspensions will not agglutinate in monospecific B antiserum. The B antigen appears to be relatively rare.

(3) The flagellar or H antigens of the coliform bacilli are often poorly developed. Some twenty-two components have been found, of which twenty are used for purposes of identification.

Kauffmann has developed a serological classification of coliform bacilli, largely based on the distribution of O, K, and H antigens. In general, about 80 per cent of strains having K antigens contain L antigens, and the other 20 per cent contain A antigen or B antigen. Strains containing K antigen appear in a general way to be the more toxic and more resistant to phagocytosis and the bactericidal action of antibody and are found more frequently in pathological material than in feces. Some of the coliform bacteria are immunologically related to some of the other gram-negative bacilli, such as the plant pathogens, Friedländer's bacillus, Salmonella, Shigella,[22] and Pseudomonas,[57] while others appear to be related to certain of the pneumococcus types.

The interesting observation has been made[45, 46, 47, 65] that while most of the colon bacilli harbored by a given individual may be immunologically identical, or nearly so, individuals show a continuous succession of types, each predominating for a few weeks or months and then being replaced by a fresh type, or serotypes may persist for extended periods. It was noted also that immunologically identical types were sometimes biochemically different.

Pathogenicity for Man. The pathogenicity of the colon bacilli in their natural habitat, the intestinal tract, is ordinarily very slight. These bacteria are associated with institutional outbreaks of infant diarrhea, and a number of serotypes are found, and may be causally related to the disease. Two were differentiated relatively early and have been given a number of designations. One of these has been called *Bact. coli neapolitanum, E. coli* D433, *E. coli* α-type, *Bact. coli* Bray

or BGT, and *E. Coli* O group 111. The other is *E. coli* β-type or *E. coli* O group 55. These O serological groups, usually designated O55 and O111, are those arbitrarily numbered by Kauffmann, and provide precise identification. *E. coli* O55 is immunologically related to the pathogenic paracolon bacilli of the Arizona group, and *E. coli* O111 to the Salmonella antigen XXXV.[34]

A considerable number of other O serotypes have now been associated with diarrheal disease, including O26, O44, O86, O112, O119, O124, O125, O126, O127 and O128. Compilation of available data[26] has shown that the serotypes, more precisely defined by inclusion of additional antigens, most often associated with diarrheal disease in this country are: O55:B5:–; O55:B5:H6; O55:B5;H7; O11:B4:–; O11:B4:H2; O11:B4:H12; and O127:B8:–. Such complete serotyping is necessary if accurate data are to be obtained.

The basis of the pathogenicity of these serotypes is uncertain, but it has been found[51] that the rabbit gut reaction, devised by De and his associates in studies of cholera (Chap. 23), and consisting of increased exudate and dilation of the gut wall of ligated sections of the small bowel, tends to be positive with enteropathogenic coliforms, and negative with those which are not associated with diarrheal disease.

The presence of these serotypes is not necessarily associated with diarrheal disease. In one study[48] of children in the 0-2 age group, for example, the asymptomatic carrier rate was 5.6 per cent. Of those infected, 41 per cent showed no symptoms, and the proportion increased with age.

The causative agents of infant diarrhea differ in various parts of the world and generally reflect economic and public health conditions. In backward countries with a high infant mortality rate due to diarrheal disease, the causative microorganisms are most commonly the Flexner and Shiga dysentery bacilli (Chap. 22), the common causes of adult dysentery. In advanced countries the dysentery bacilli, and to a considerable extent Salmonella, are less common causes of infant diarrhea, and are replaced by coliforms.[43] The picture in this country is similar to, for example, that observed in England[50] and France.[38]

The laboratory diagnosis of diarrheal disease of coliform etiology has been systematized[44] and, as indicated above, involves detailed serotyping. A rapid presumptive identification of enteropathogenic coliforms in rectal swabs, using the fluorescent antibody technique, has been worked out in detail[5, 52, 53] and appears to be very promising.

Outside the intestinal tract, the colon bacilli have definite pathogenic potential-

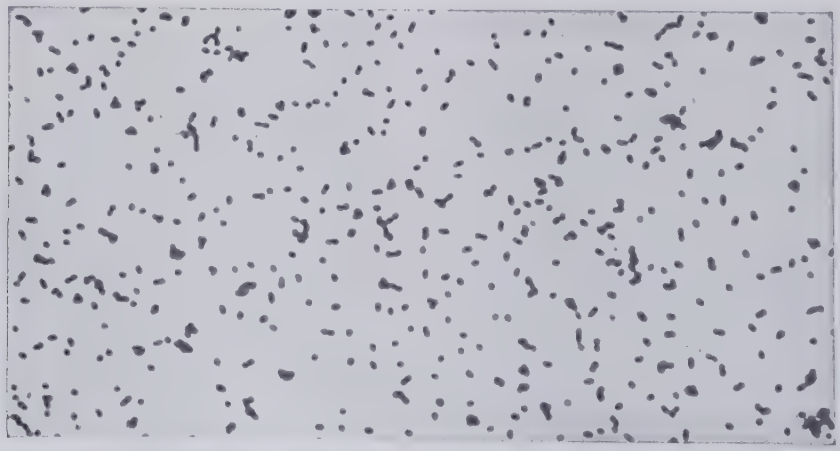


Figure 101. Coliform bacilli. Smear from a pure culture. Note the coccobacillary form. Fuchsin; × 1050.

ities. The urinary tract is probably the most frequently invaded, and the majority of cases of cystitis are of coliform etiology. The colon bacilli may also play a part in the formation of gallstones; the bacillus is frequently found in the core of gallstones and, in culture, can precipitate cholesterol and other biliary constituents. The injection of colon bacilli into the healthy urinary bladder or gallbladder does not produce infection in experimental animals, but infection occurs if the bile duct or urethra is obstructed. Local infections such as abscesses, conjunctivitis, and the like, have been observed but are not common. *E. coli* septicemia is very rare but may occur as an agonal invasion in acute infective processes. A hemorrhagic septicemia caused by *E. coli* sometimes occurs in newborn infants and is known as Winckel's disease.

Pathogenicity for Lower Animals. *E. coli* is of low pathogenicity for laboratory animals. Two milliliters of a broth culture injected intraperitoneally will kill a guinea pig within a few days, and killed bacilli are very nearly as effective. Spontaneous infection of lower animals is not common. The diarrhea of young calves known as scours has been attributed to *E. coli* septicemia. The colon bacillus is also thought to be a factor in the causation of diarrhea of foals and young pigeons. Local infection may occur; in one series of 286 cases of bovine mastitis, ten were apparently due to members of the colon-aerogenes group, of which three were typical *E. coli.*

FRIEDLÄNDER'S BACILLUS (KLEBSIELLA PNEUMONIAE)

The microorganism described by Friedländer as an etiological agent of pneumonia is, as indicated above, nonmotile and, as found in the respiratory tract, usually heavily encapsulated. Motile strains occur in such pneumonias, however, and strictly speaking they are not *Klebsiella pneumoniae,* but the distinction is not relevant to the disease produced. Occasional strains are pleomorphic in culture. Friedländer's bacillus is readily isolated from sputum from cases of the pneumonia caused by it by culture on blood agar. The growth is heavy and vicous, and the colonies unusually large. For practical purposes, this kind of growth and demonstration of the gram-negative, thick, ovoid rods, often occurring in pairs, suffices for identification.

Figure 102. *Klebsiella pneumoniae* (Friedländer's bacillus) in pure culture on blood agar, showing capsules. Crystal violet; × 1200.

As indicated earlier, these bacteria are extremely closely related to the aerogenes type of coliform. Differentiation may be exceedingly difficult[41] if not practically impossible at times, and is irrelevant to the bacteriological diagnosis of pneumonia. Detailed examination of strains allows differentiation within Klebsiella into *K. pneumoniae, K. ozaenae* and *K. rhinoscleromatis,* the last two being associated with a type of chronic upper respiratory tract infection, together with certain other types given species names.[9, 33]

Immunological Types. This group of bacteria, including both motile and nonmotile forms, contains three kinds of antigens, designated O, A, and K, and analogous to those of *E. coli.* The bacteria are typed on the basis of the cap-

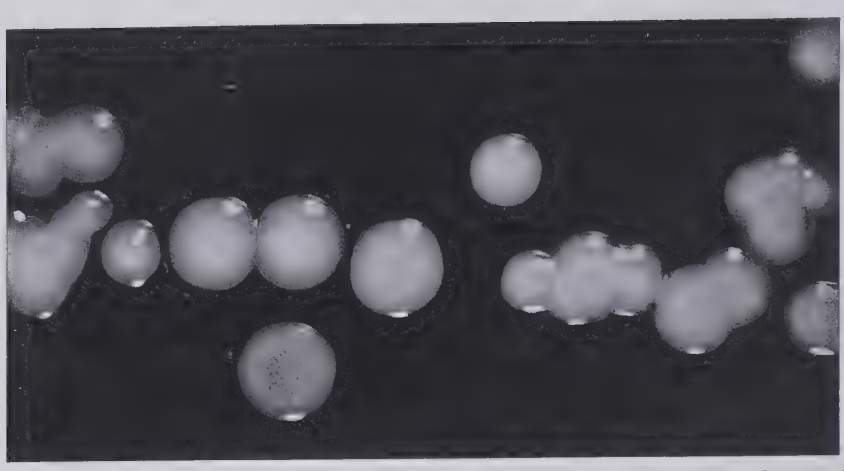


Figure 103. Colonies of Friedländer's bacillus on blood agar. Note the large size and mucoid appearance. × 3.

sular antigen, and a total of 57 capsular types have been described.[12, 13] The Quellung reaction does not occur in specific antiserum, but a precipitin reaction takes place at the periphery of the capsule, making the margin highly refractile. There appears to be no variation in virulence associated with capsular type.

Pathogenicity. Microorganisms of this group are found associated with various kinds of upper respiratory disease in man, and in most instances are probably secondary invaders, as in the nasopharynx in persons having chronic sinusitis or chronic lung infections such as bronchiectasis.

Pneumonia due to Friedländer's bacillus makes up 0.5 to 4.0 per cent of all pneumonia, but the case fatality rate is high, 90 per cent or more in untreated cases. The microorganism has a greater tendency to produce necrotic lesions than pneumococcus, and the infection contrasts with pneumococcal pneumonia in that while it is similar in its very early stages with a spreading zone of infected edema around an older area of dense exudate, as the infection progresses the alveolar walls become involved and disintegrate, and multiple areas of purulent exudate become necrotic. Thus the pathological response is necrosis of the lung parenchyma and subsequent healing with fibrosis, commonly accompanied by abscess formation, cavitation, or bronchiectasis. The sulfonamides and broad spectrum antibiotics, but not penicillin, are reasonably effective, and according to some workers are more effective in combination.

Probably antimicrobial treatment instituted very early would result in complete recovery, but the infected individual is usually not hospitalized until the second day, and another day is wasted with penicillin therapy. In the drug-arrested infection, the affected areas become a lesion consisting of one or more cavities lined with necrotic material connected to damaged bronchi, and chemotherapy transforms the acute, often fatal, condition to a subacute or chronic infection.

Despite chemotherapy the residual lung damage frequently requires surgical drainage and later pulmonary resection. During surgery there is some likelihood of a flare-up of infection in the contralateral lung due to persistence and drug resistance of the infecting microorganism.[31, 58]

Klebsiella has also been associated from time to time with suppurative conditions in various parts of the body, such as liver abscess and meningitis,[54] and rarely has been found to invade the blood stream to produce septicemia. Infection may also occur spontaneously in lower animals, and these bacteria have been found to be the etiological agents of epidemic respiratory infection in mice, a paralytic disease of moose, and metritis of mares.

THE PARACOLON BACILLI

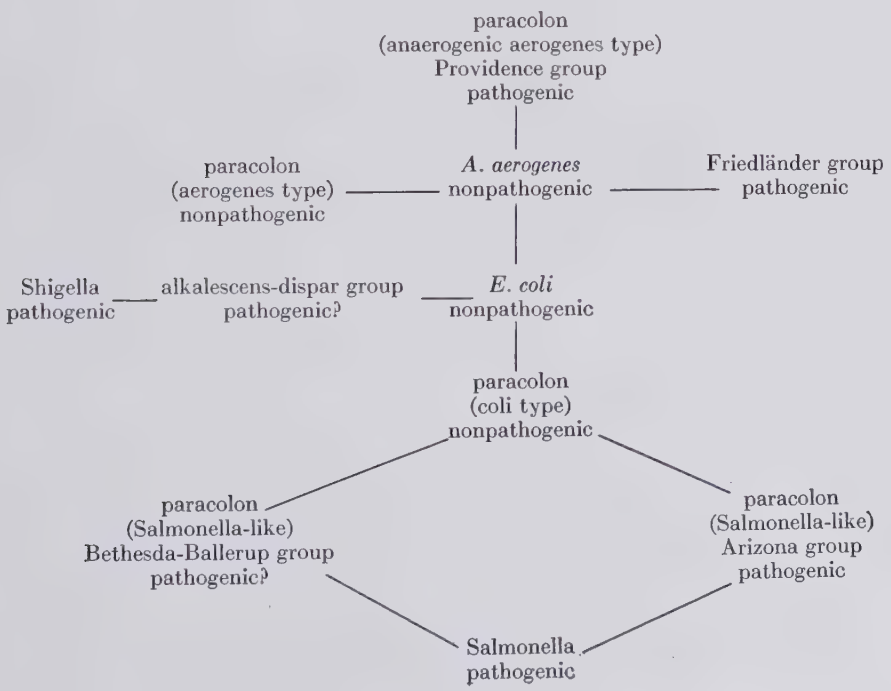
The term paracolon bacillus is applied to enteric bacilli similar to the colon-aerogenes group, but distinguished from it by a delayed (5 to 21 days) fermentation of lactose. On the basis of the IMViC reactions, these slow lactose fermenters range from a typical coli type to a typical aerogenes type. Certain biochemical types of paracolon bacilli closely resemble the Salmonellas and, especially when the lactose fermentation is long delayed, may present a practical problem in reasonably rapid identification.

Study of the serological character of some of these forms, especially those that are found in apparent etiological relationship to enteric disease, has shown that they are related on the one hand to coliform bacilli and on the other to Salmonella, and some antigenic components are shared with certain of the dysentery bacilli. Still others are more closely related to Proteus. Their division into groups is more a matter of convenience for purposes of study than a recognition of sharply defined differences among them. A number of such groups are generally recognized;[10] a rough approximation of their relationship to one another and to other enteric bacilli, arrived at only by disregard of some apparently minor resemblances, is shown in the accompanying diagram.

The Ballerup-Bethesda Group.[29, 56] The paracolon bacilli in this group closely resemble the Salmonellas in the usual biochemical characteristics except that they ordinarily, but not invariably, ferment lactose and sucrose, and often salicin, on prolonged incubation. They were originally regarded as two separate types, one of which was described as *Salmonella ballerup* though it was immunologically related to the other Salmonella types only through its content of Vi antigen.

The first of the Bethesda group of paracolon bacilli were originally isolated in Bethesda, Maryland, but subsequently

Approximate Interrelationships of the Enteric Bacilli



they have been found widely scattered over the country. Many of the Ballerup strains are late lactose and/or sucrose fermenters and are now regarded as paracolons, and have been merged with the Bethesda group since the two are closely related antigenically. So far thirty-two O groups and seventy-four H antigens are recognized and occur in various combinations to give a multiplicity of serological types. These bacteria have been isolated with some frequency in association with enteric disease, and at least some strains are possible pathogens. They are often encountered in diagnostic work and are not rapidly separable from Salmonella by biochemical tests; pooled and polyvalent antiserums facilitate their recognition.

The Arizona Group.[14, 15] These bacteria were first described as Salmonella found in reptiles and resemble the Salmonellas closely except that most strains ferment lactose within two weeks. They are closely related serologically to Salmonella; many of the O antigens are identical with those of Salmonella, and the H antigens are closely similar. These bacteria fall into seventy-six O groups, and seventy-five serological types have been described. It appears to be definitely established that this group of paracolon bacilli is regularly pathogenic, not only for cold-blooded animals, but also for fowls and man, and in the last two produces infections as severe and fatal as do the Salmonellas.

The Providence Group.[16] This group is made up of paracolon bacilli of an aerogenes type (citrate-positive) that are anaerogenic, *i.e.*, produce little or no gas from glucose. They were originally described as the 29911 group but are now known as the Providence* group. They are closely related to Proteus, so much so that it has been suggested that they be considered as a species of Proteus, *Pr. inconstans*. They have been associated with institutional outbreaks of diarrhea among infants and may be regarded as possible pathogens. Their serology is as yet incompletely worked out, and this has hampered their identification in association with enteric disease.

PROTEUS[16]

These microorganisms were originally described by Hauser as an independent genus containing three species—*Pr. vulgaris*, *Pr. mirabilis*, and *Pr. zenkeri*. It is now generally agreed that the last is not closely related to the typical members of the group (it is gram-positive), and it is now placed in a separate genus as *Kurthia zenkeri*. As indicated earlier, Proteus is included under the Enterobacteriaceae and is related to but nevertheless distinct from the other enteric bacilli. It is found with some frequency in normal feces and often increases proportionately during or immediately after attacks of diarrheal disease caused by other organisms. It is one of the most common bacteria in soil and water containing decaying organic matter of animal origin and usually occurs in large numbers in sewage; it is perhaps to be identified with the "bacterium of putrefaction" or *Bact. termo* of early writers. Like the enteric pathogens it does not ferment lactose, and it resembles them in its growth on differential and selective mediums. It may be confused with Salmonella because of its motility and gas formation during carbohydrate fermentation, but is distinguished by its ability to hydrolyze urea.[6]

Morphology and Staining. In general, these bacteria appear as straight or slightly curved rods 1 to 2.5 μ in length and 0.4 to 0.6 μ in breadth, frequently in end-to-end pairs and short chains. Ovoid forms are common, however, and long, curved, filamentous cells predominate in actively swarming cultures. Proteus is

* They were originally described and have been studied most intensively by Stuart and his co-workers at Brown University in Providence, Rhode Island.

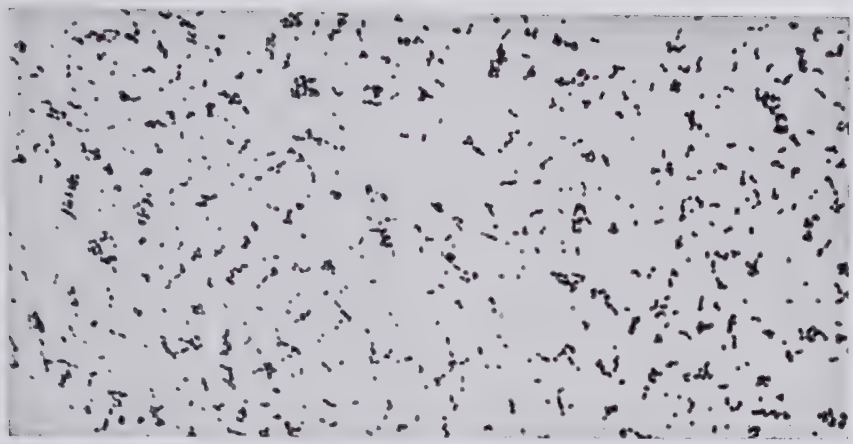


Figure 104. *Proteus vulgaris.* Smear from a pure culture. Note the coccobacillary form. The occasional occurrence of paired cells is a result of active multiplication. Fuchsin; × 1050.

actively motile by peritrichous flagella and forms neither capsules nor spores.

The phenomenon of "swarming" exhibited by these bacilli is a consequence of their active motility. On the surface of agar mediums the colonies do not remain compact and discrete; the growth spreads rapidly over the entire surface available as a thin, scarcely visible bluish film. Microscopic observation shows that the bacilli break away from the edge of the growth and migrate or "swarm" over the surface of the medium, thus giving rise to the thin film of growth. This property is a source of considerable inconvenience in the isolation of bacteria other than Proteus from mixed cultures in which it is present; the inclusion of 0.01 per cent sodium azide inhibits the growth of this and other gram-negative bacilli but allows the growth of streptococci.

These bacilli stain readily with the usual aniline dyes and are gram-negative.

Physiology. The nutritive requirements of Proteus are simple, and the bacilli grow readily upon the ordinary laboratory mediums. They may be cultivated in synthetic solutions containing ammonium lactate, but nicotinic acid must be supplied. The optimum temperature for growth is 30° to 37° C., though good growth occurs at 20° C. These bacteria are facultative anaerobes,

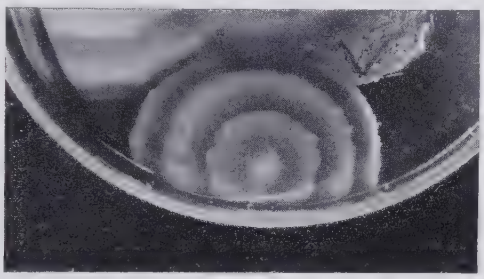


Figure 105. *Proteus vulgaris* colony on blood agar. Note the swarming exhibited as successive waves of growth. (Dack.)

but anaerobic growth is generally very poor.

Gelatin is liquefied more or less rapidly, often with a characteristic colony formation with radiating filaments which wander far off into the surrounding medium. The typical Proteus colonies are best formed when the gelatin is soft, as happens when it is kept at a temperature not far below the melting point or is made up with 5 instead of 10 per cent gelatin. Dextrose is fermented with acid and gas production; sucrose and maltose are fermented by some, but not by all, strains; lactose, raffinose, and mannitol are never fermented by the typical *Pr. vulgaris.* The Voges-Proskauer reaction is negative and the methyl red test positive. Nitrates are reduced. Milk is at first rendered slightly acid, then curdled with alkaline reaction (in about 3 days), and more or less slowly peptonized. While

species of Proteus other than *Pr. morgani* are actively proteolytic, these bacteria apparently do not play an important part in the anaerobic decomposition of proteins or putrefaction as was once supposed. Under aerobic conditions proteolysis occurs rapidly, *i.e.*, decay or nonputrefactive proteolysis.

At the present time the bacteria comprising the genus Proteus are classified entirely on a biochemical basis.[25] As noted above, the group is distinguished by its ability to hydrolyze urea. Four species are recognized: *Pr. vulgaris, Pr. mirabilis, Pr. rettgeri,* and *Pr. morgani;* the last is known in the earlier literature as Morgan's bacillus (see below). These are distinguished from one another on the basis of the fermentation of mannitol, maltose, and sucrose and the formation of indol.

Antigenic Structure. Proteus contains both H and O antigens when motile, and the nonmotile, nonswarming strains contain only O antigen. Three colonial types have been observed,[12] and antigenic analysis of the O and H antigens has given eighteen O groups in forty-four strains, with recurring and crossing of H antigens and some suggestion of phase variation.[13] In general, however, the serology of Proteus is incomplete.

Certain Proteus strains are agglutinated by the serum of patients having typhus fever. These so-called X strains contain an antigen common to the typhus rickettsiae, and the agglutination of these bacilli (the Weil-Felix reaction) is of diagnostic value in typhus fever. In the Proteus strains the antigen is a part of the O antigen, and its specificity is determined by an alkali-stable carbohydrate haptene which is also found in *Rickettsia prowazeki.* The X strains frequently ferment maltose.

Pathogenicity. Proteus, both in mixed and pure cultures, has been found to be associated with a variety of pathological conditions. Infections of the eye and ear, pleuritis and peritonitis and suppurative abscesses in many parts of the body are among the many instances in which the pathogenic power of this bacterium in pure culture can hardly be doubted. As a producer of cystitis and pyelonephritis it ranks next to *E. coli.* There is evidence[4] that Proteus urease is nephrotoxic, favoring the intracellular infection of the tubular epithelium, and creating an alkalinity in the kidney which leads to necrosis of renal tubular epithelium with the precipitation of $MgHPO_4$ with the formation of stones. Besides its independent pathogenicity, it is found so commonly associated with other microorganisms in purulent war wounds and similar processes that its activity as an accomplice is probably second only to that of the cocci.

In certain affections of the digestive tract Proteus has been frequently held to be the responsible agent. In diarrheic stools, especially those of infants, it has often been found in large numbers and is regarded by many as a cause of infant diarrhea. The real relation of Proteus to intestinal infection is, however, still obscure. Certain food-poisoning epidemics have been ascribed to Proteus.

Animal inoculation shows that a great range of virulence exists among the Proteus cultures that have been tested. Freshly isolated strains from pathological sources may produce definite lesions, including abscesses, enlargement of the spleen, and a diarrheic condition. The etiological agent of red-leg disease of frogs, ulcer disease of brook trout, and red sore of pike has been found to be a bacillus closely related to the Proteus group, and it has been classified as *Pr. hydrophilus,*[37, 40] and is not to be confused with Aeromonas species, which are also pathogenic for cold-blooded animals to produce similar diseases. The cell substance of these bacteria is toxic on parenteral injection and, as in the case of the enteric forms, appears to be a glucolipid. No difference in toxicity is apparent between strains of pathogenic and nonpathogenic origin.

Morgan's Bacillus. This bacillus was isolated by Morgan in 1906 from the stools of infants suffering from summer diarrhea. Although it was first designated as Morgan's No. 1, the other varieties of Morgan's bacilli have long since faded out of general interest and recognition so

that the name Morgan's bacillus is usually applied to Morgan's No. 1. It is closely related to the Proteus group, and it is classified as *Pr. morgani*. This bacillus appears to have played a part in a number of outbreaks of summer diarrhea of infants and has been isolated from paratyphoid-like fevers. It has been found to give rise to spontaneous epidemics of enteritis in mice. Injected intraperitoneally into mice, Morgan's bacillus produces a rapidly fatal infection.

REFERENCES

1. Bamforth, J., and Dudgeon, J. A.: The haemolytic activity of *Bact. coli*. J. Path. Bact., 1952, **64**:751–761.
2. Belyavin, G.: Cultural and serological phases of *Proteus vulgaris*. J. Gen. Microbiol., 1951, **5**: 197–207.
3. Belyavin, G., Miles, E. M., and Miles, A. A.: The serology of fifty strains of *Proteus vulgaris*. J. Gen. Microbiol., 1951, **5**:178–196.
4. Braude, A. I., and Siemienski, J.: Role of bacterial urease in experimental pyelonephritis. J. Bact., 1960, **80**:171–179.
5. Cherry, W. B., Thomason, B. M., Pomales-Lebron, A., and Ewing, W. H.: Rapid presumptive identification of enteropathogenic *Escherichia coli* in faecal smears by means of fluorescent antibody. 3. Field evaluation. Bull. W.H.O., 1961, **25**:159–171.
6. Cook, G. T.: Urease and other biochemical reactions of the Proteus group. J. Path. Bact., 1948, **60**:171–181.
7. Cowan, S. T.: Taxonomic rank of Enterobacteraceae 'groups.' J. Gen. Microbiol., 1956, **15**:345–358.
8. Cowan, S. T.: Nomenclature for Enterobacteraceae. Bull. Hyg., 1957, **32**:101–103.
9. Cowan, S. T., Steel, K. J., Shaw, C., and Duguid, J. P.: A classification of the Klebsiella group. J. Gen. Microbiol., 1960, **23**:601–612.
10. Edwards, P. R., and Ewing, W. H.: Status of serologic typing in the family Enterobacteraceae. Amer. J. Pub. Health, 1952, **42**:665–671.
11. Edwards, P. R., and Ewing, W. H.: Identification of Enterobacteraceae. Burgess, Minneapolis. 2nd ed., 1962.
12. Edwards, P. R., and Fife, M. A.: Capsule types of Klebsiella. J. Infect. Dis., 1952, **91**:92–104.
13. Edwards, P. R., and Fife, M. A.: Studies on the Klebsiella-Aerobacter group of bacteria. J. Bact., 1955, **70**:382–390.
14. Edwards, P. R., Fife, M. A., and Ramsey, C. H.: Studies on the Arizona group of Enterobacteraceae. Bact. Rev., 1959, **23**:155–174.
15. Edwards, P. R., McWhorter, A. C., and Fife, M. A.: The Arizona group of Enterobacteraceae in animals and man. Occurrence and distribution. Bull. W.H.O., 1956, **14**:511–528.
16. Ewing, W. H.: The nomenclature and taxonomy of the Proteus and Providence groups. Internat. Bull. Bact. Nomen. Taxon., 1958, **8**:17–23.
17. Ewing, W. H.: Enterobacteraceae. Biochemical Methods for Group Differentiation. Communicable Disease Center, Public Health Service, U. S. Department of Health, Education, and Welfare. 1960.
18. Ewing, W. H., and Davis, B. R.: The O antigen groups of *Escherichia coli* cultures from various sources. Communicable Disease Center, Public Health Service, U. S. Department of Health, Education and Welfare. 1961.
19. Ewing, W. H., Davis, B. R., and Edwards, P. R.: The decarboxylase reactions of Enterobacteraceae and their value in taxonomy. Pub. Health Lab., 1960, **18**:77–83.
20. Ewing, W. H., Davis, B. R., and Reavis, R. W.: Studies on the Serratia group. Communicable Disease Center, Public Health Service, U. S. Dept. of Health, Education and Welfare. 1959.
21. Ewing, W. H., and Edwards, P. R.: The principal divisions and groups of Enterobacteraceae and their differentiation. Internat. Bull. Bact. Nomen. Taxon., 1960, **10**:1–12.
22. Ewing, W. H., Hucks, M. C., and Taylor, M. W.: Inter-relationships of certain Shigella and Escherichia cultures. J. Bact., 1952, **63**:319–325.
23. Ewing, W. H., Hugh, R., and Johnson, J. G.: Studies on the Aeromonas group. Communicable Disease Center, Public Health Service, U. S. Department of Health, Education and Welfare. 1961.
24. Ewing, W. H., and Johnson, J. G.: The differentiation of Aeromonas and C27 cultures from Enterobacteraceae. Internat. Bull. Bact. Nomen. Taxon., 1960, **10**:223–230.
25. Ewing, W. H., Suassuna, I., and Suassuna, I. R.: The biochemical reactions of members of the genus Proteus. Communicable Disease Center, Public Health Service, U. S. Department of Health, Education and Welfare. 1960.
26. Ewing, W. H., Tatum, H. W., and Davis, B. R.: The occurrence of *Escherichia coli* serotypes associated with diarrheal disease in the United States. Pub. Health Lab., 1957, **15**:118–138.
27. Ewing, W. H., Tatum, H. W., Davis, B. R., and Reavis, R. W.: Studies on the serology of the *Escherichia coli* group. Communicable Disease Center, Public Health Service, U. S. Department of Health, Education and Welfare. 1956.
28. Fulton, M., Forney, C. E., and Leifson, E.: Identification of Serratia occurring in man and animals. Canadian J. Microbiol., 1959, **5**:269–275.
29. Galbraith, E. L., and Crossley, V. M.: The Bethesda group of paracolon bacteria. Canadian J. Pub. Health, 1953, **44**:172–176.
30. Hormaeche, E., and Edwards, P. W.: A proposed genus Enterobacter. Internat. Bull. Bact. Nomen. Taxon., 1960, **10**:71–74.
31. Jervey, L. P., and Hamburger, M.: The treatment of acute Friedländer's bacillus pneumonia. A continuing problem. Arch. Intern. Med., 1957, **99**:1–7.
32. Kauffmann, F.: Review: The serology of the coli group. J. Immunol., 1947, **57**:71–100.
33. Kauffmann, F.: The Differentiation of Escherichia and Klebsiella Types. Thomas, Springfield, Illinois. 1951.
34. Kauffmann, F.: On antigenic relationships be-

tween Escherichia strains from infantile enteritis and Salmonella or Arizona strains. Acta Path. Microbiol. Scand., 1952, **31:**355–359.

35. Kauffmann, F., Edwards, P. R., and Ewing, W. H.: The principles of group differentiation within the Enterobacteraceae by chemical methods. Internat. Bull. Bact. Nomen. Taxon., 1956, **6:**29–33.
36. Kauffmann, F., and Petersen, A.: The biochemical group and type differentiation of Enterobacteraceae by organic acids. Acta Path. Microbiol. Scand., 1956, **38:**481–491.
37. Kulp, W. L., and Borden, D. G.: Further studies on *Proteus hydrophilus,* the etiological agent in "red leg" disease of frogs. J. Bact., 1942, **44:**673–685.
38. Le Minor, S., Le Minor, N. P., and Buttiaux, R.: Êtudes sur les *Escherichia coli* isolés au cours des gastro-entérites infantiles. I. Propriétés biochimiques et antigéniques. Ann. Inst. Pasteur, 1954, **86:**204–226.
39. Moeller, V.: Simplified tests for some amino acid decarboxylases and the arginine dihydrolase system. Acta Path. Microbiol. Scand., 1955, **36:**158–172.
40. Reed, G. B., and Toner, G. C.: *Proteus hydrophilus* infections of pike, trout and frogs. Canadian J. Res., C, 1942, **20:**161–166.
41. Renoux, G., and Terdjman, A.: *Aerobacter aerogenes* et *Klebsiella pneumoniae.* Ann. Inst. Pasteur, 1952, **82:**242–245.
42. Report: Report of the Enterobacteraceae Subcommittee of the Nomenclature Committee of the International Association of Microbiological Societies. Internat. Bull. Bact. Nomen. Taxon., 1958, **8:**25–70.
43. Rodríguez-Leiva, M.: *Escherichia coli* studies. VII. Incidence of enteropathogenic *Escherichia coli* in the acute diarrhea of infants in Santiago, Chile. Amer. J. Hyg., 1960, **72:**162–168.
44. Rogers, K. B., and Taylor, J.: Laboratory diagnosis of gastro-enteritis due to *Escherichia coli.* Bull. W. H. O., 1961, **24:**59–72.
45. Sears, H. J., et al.: Persistence of individual strains of *Escherichia coli* in man and dog under varying conditions. J. Bact., 1956, **71:** 370–372.
46. Sears, H. J., and Brownlee, I.: Further observations on the persistence of individual strains of *Escherichia coli* in the intestinal tract of man. J. Bact., 1952, **63:**47–57.
47. Sears, H. J., Brownlee, I., and Uchiyama, J. K.: Persistence of individual strains of *Escherichia coli* in the intestinal tract of man. J. Bact., 1950, **59:**293–301.
48. Soloman, P., Weinstein, L., and Joress, S. M.: Studies of the incidence of carriers of enteropathogenic *Escherichia coli* in a pediatric population. J. Pediatrics, 1961, **58:**716–721.
49. Suassuna, I., Suassuna, I. R., and Ewing, W. H.: The methyl red and Voges-Proskauer reactions of Enterobacteraceae. Pub. Health Lab., 1961, **19:**67–74.
50. Taylor, J.: The diarrhoeal diseases in England and Wales with special reference to those caused by Salmonella, Escherichia and Shigella. Bull. W.H.O., 1960, **23:**763–779.
51. Taylor, J., Wilkins, M. P., and Payne, J. M.: Relation of rabbit gut reaction to enteropathogenic *Escherichia coli.* Brit. J. Exptl. Path., 1961, **42:**43–52.
52. Thomason, B. B., Cherry, W. B., Davis, B. R., and Pomales-Lebron, A.: Rapid presumptive identification of enteropathogenic *Escherichia coli* in faecal smears by means of fluorescent antibody. I. Preparation and testing of reagents. Bull. W.H.O., 1961, **25:**137–152.
53. Thomason, B. M., Cherry, W. B., and Pomales-Lebron, A.: Rapid presumptive identification of enteropathogenic *Escherichia coli* in faecal smears by means of fluorescent antibody. 2. Use of various types of swabs for collection and preservation of faecal specimens. Bull. W.H.O., 1961, **25:**153–158.
54. Thompson, A. J., Williams, E. B., Jr., Williams, E. D., and Anderson, J. M.: *Klebsiella pneumoniae meningitis.* Review of the literature and report of a case with bacteremia and pneumonia, with recovery. Arch. Intern. Med., 1952, **89:**405–420.
55. Wallick, H., and Stuart, C.: Antigenic relationship of *Escherichia coli* isolated from one individual. J. Bact., 1943, **45:**121–126.
56. West, M. G., and Edwards, P. R.: The Bethesda-Ballerup group of paracolon bacilli. Communicable Disease Center, Public Health Service, U. S. Department of Health, Education and Welfare. Publication No. 362, 1954.
57. Wiedermann, G., and Flamm, H.: Antigengemeinschaft zwischen Pseudomonas und Escherichia. Zent. f. Bakt., I Abt., Orig., 1961, **182:**67–70.
58. Wylie, R. H., and Korschner, P. A.: Friedländer's pneumonia. Amer. Rev. Tuberc., 1950, **61:** 465–473.

Chapter 21

THE ENTERIC BACILLI

The Salmonella Group

THE ENTERIC bacilli comprising the large Salmonella group are all pathogenic to a greater or lesser degree and include the typhoid bacillus, the paratyphoid bacilli, and a wide variety of forms whose natural hosts are lower animals, especially rodents and birds.

The infectious nature of typhoid fever was apparent in 1856 to William Budd, who, on the basis of epidemiological evidence, suggested that the disease was transmitted by sewage-contaminated water and that the source of the infectious agent was human feces. The typhoid bacillus was found by Eberth in 1880 in the mesenteric glands and spleen of persons dying from typhoid fever and was cultured in 1884 by Gaffky.

The rodent pathogen, *Salmonella enteritidis*, was described in 1888 by Gärtner who isolated it from infected beef responsible for an outbreak of gastroenteritis, and in the older literature this microorganism is known as Gärtner's bacillus.

For a good many years the kinds of microorganisms making up the "typhoid-paratyphoid group" were relatively few and confined largely to those differentiable by cultural reactions. With the development of the techniques of antigenic analysis and their application to these microorganisms in the 1920's, very many serologically differentiable types or kinds were, and continue to be, described until by now the Salmonella group is made up of many hundreds of differentiable types.[30, 60]

Morphology and Staining. These bacilli are gram-negative rods closely resembling and indistinguishable from the coliform bacteria. They stain readily with the usual dyes such as methylene blue and carbol-fuchsin. No particular arrangement of the cells is apparent on microscopic examination. All species except *Sal. pullorum* and *Sal. gallinarum* are actively motile by means of peritrichous flagella. No capsules are apparent, and spores are not formed.

Physiology. The bacteria of this group have simple nutritional requirements, growing readily on the usual nutrient mediums. In synthetic mediums an ammonium salt and glucose, pyruvate, lactate, etc., are adequate sources of nitrogen and carbon. The great majority of strains do not require bacterial vitamins or amino acids, but some strains of the typhoid bacillus require added tryptophan. The optimum temperature is 37° C., but growth occurs at a reasonable rate at room temperature. They are facultative anaerobes, growing equally well under either aerobic or anaerobic conditions, and some species develop relatively strong reducing tendencies.

The group is characterized biochem-

ically by failure to ferment lactose or salicin and inability to liquefy gelatin or produce indol. There are a very few exceptions to the two last; *Sal. eastbourne* and some strains of *Sal. enteritidis* and *Sal. panama* produce indol, and *Sal. daressalaam* liquefies gelatin. The evolution of gas commonly accompanies the fermentation of sugars, though anaerogenic strains of *Sal. enteritidis, Sal. typhimurium,* and *Sal. paratyphi C* have been reported. *Sal. typhi,* however, characteristically ferments sugars without the formation of gas.

A great variety of cultural reactions, not only the conventional sugar fermentations, formation of indol from tryptophan, etc., but various kinds of specialized tests, such as the utilization of the tartaric acids and malonate, amino acid decarboxylase activity, etc. have been useful in the physiological characterization of bacteria of this group.[27, 29]

Toxins. Like the other enteric bacilli, the Salmonellas do not form exotoxins but all contain endotoxins. These are the polysaccharide-polypeptide-lipid complexes extractable from the intact cells with trichloracetic acid or glycols and are extracted as toxic lipopolysaccharide in 50 per cent phenol. They appear to occur largely in the cell wall of the bacteria. These endotoxins, together with those of the coliform bacilli, have been taken as prototypes and most of the information available on endotoxins (Chap. 9) has been derived from them.

The toxicity is nonspecific in the sense that endotoxins from whatever source produce substantially the same reactions on parenteral inoculation. The more obvious responses are fever and alteration in capillary permeability, the former probably contributing to the symptoms of the disease. The endotoxins are antigenic, stimulating the formation of agglutinating, precipitating, and protective antibody activity, but the antitoxic activity of antibody is of a low order. The specificity is that of the contained polysaccharide, and the endotoxins are apparently identical with the heat-stable somatic antigenic complex of the bacteria.[52, 89]

Another toxic substance, extractable in alcohol and named the Q substance, has been found in typhoid bacilli and other enteric bacilli, but its relation to the endotoxin is uncertain.

Immunological Differentiation. As indicated above, the techniques of antigenic analysis have been developed in the study of the Salmonella group, and the antigenic composition of bacteria of this group is, perhaps, better known than that of any other bacteria. Many members of the group are immunologically complex; whether because they are innately so or because they are better known in this respect than most other bacteria is difficult to say.

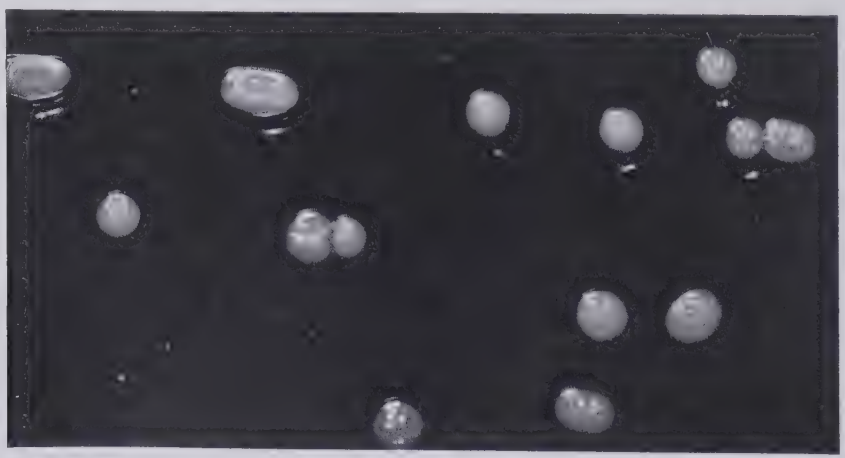


Figure 106. Colonies of *Salmonella typhimurium* (*aertrycke*) on nutrient agar. Twenty-four-hour culture. × 3.

Flagellar and somatic antigens. Two types of antigens are present in the bacteria of the Salmonella group, one associated with the cell substance and the other with the flagella. Noted by Smith and Reagh in 1903, the first was termed *somatic antigen* and the second *flagellar antigen*. These types were later observed by Weil and Felix who designated them O and H antigens respectively. The O antigens are arbitrarily designated by Roman numerals.

These two types of antigen, each of which may be, and frequently is, represented in a single bacterial strain by more than one component, differ from one another in several respects. The flagellar antigen is the more unstable and is destroyed by boiling and by exposure to alcohol or weak acid; somatic antigen, on the other hand, is stable to boiling, alcohol, and acid. Stock somatic antigen for immunization purposes may be prepared by heating at 100° C., followed by treatment with alcohol and drying with acetone.[22] Cultures on phenol agar (0.1 per cent) of bacteria normally containing both H and O antigens are found to contain only O antigens, the formation of flagellar antigen having been suppressed; H antigen reappears immediately on cultivation on nutrient agar. In the agglutination reaction, bacteria lacking flagellar antigen are characteristically clumped in a finely granular precipitate (O agglutination), while bacteria containing flagellar antigen are agglutinated in a coarse, flocculent precipitate (H agglutination).

The H and O agglutination titer of an antiserum may be determined by the use of H and O antigens in the agglutination test. H antigen is commonly prepared by adding an equal volume of formol (0.6 per cent formalin) saline to an 18- to 24-hour broth culture. In the preparation of somatic antigen the flagellar component is destroyed by treatment with alcohol; the growth from an 18- to 24-hour agar slant culture is emulsified in 1 to 2 ml. of absolute alcohol, heated at 60° C. for 1 hour, centrifuged, and the sediment suspended in 0.5 to 1 ml. saline. It may be used for slide agglutination or appropriately diluted for macroscopic agglutinin titrations.

These two types of antigen are immunologically independent, and the immunization of an animal with a microorganism containing both results in the production of antibodies to both. There is, however, a marked difference in titer, for the O antibody titer is generally much lower than that of the H antibody; in the writer's laboratory antiserums having H titers of 1:20,000 to 1:50,000 have shown O titers of 1:2000 or less.

Specific and Nonspecific Flagellar Antigens. The flagellar antigen is, in turn, of dual nature. One kind, designated as *specific flagellar antigen,* is individualistic and contributes in no small part to the immunological identity of a given Salmonella species. The other, termed *nonspecific flagellar antigen,* is made up of a limited number of components which are frequently shared by the various Salmonellas and hence contribute to the immunological relationship between the species of these bacteria. The specific flagellar antigens are arbitrarily designated by lower case letters (the choice was unfortunate since the more recently discovered antigens are designated z_1, z_2, z_3, etc.), and the nonspecific ones by Arabic numerals. Strains containing both kinds of H antigens are called diphasic, and those containing only either one or the other are called monophasic.

Vi Antigens. A somatic antigen, closely similar to, but not identical with, the O antigen complex, and designated Vi antigen, occurs in *Sal. typhi, Sal. paratyphi A, Sal. paratyphi C,* and Ballerup strains of paracolon bacilli.[32, 33, 34] It resembles the O antigens in that it is present in the cell substance of the microorganisms and is extractable with trichloracetic acid but is more labile to heat in the presence of water and to treatment with dilute alkali.[5, 48, 49, 63, 88, 91] It is present in practically all strains of the typhoid bacillus on primary isolation, but in only some strains of para A and para C, and has the same immunological specificity in all the microorganisms in which it oc-

curs. It was named Vi antigen, or "virulence antigen," because its presence is believed by some to be associated with virulence, and antibody to it is protective.[37]

Antigenic Structure. The components of the somatic and flagellar antigenic mosaics of bacteria of this group have been worked out in detail by application of the methods of antigenic analysis, *i.e.*, reciprocal agglutinin absorption (Chap. 14). Each of the serological types of Salmonella may be defined in immunological terms as antigenic formulas; representative antigenic formulas are shown in the accompanying table.

Representative Antigenic Formulas

O GROUP	SPECIES	ANTIGENIC FORMULA
D	*Sal. typhi*	IX, XII, (Vi): d: –
A	*Sal. paratyphi A*	I, II, XII: a: –
B	*Sal. paratyphi B*	I, IV, V, XII: b: 1, 2
C_1	*Sal. paratyphi C*	VI, VII, (Vi): c: 1, 5
C_1	*Sal. cholerae-suis*	VI, VII: c: 1, 5
B	*Sal. typhimurium*	I, IV, V, XII: i: 1, 2
D	*Sal. enteritidis*	I, IX, XII: g, m: –

This kind of pattern is known as the Kauffmann-White schema.[47] There is some association between antigenic entities in that they tend to appear in recurring combinations, and there is evidence which suggests that certain of the immunologically separable activities, *i.e.*, by absorption of antibody, may be closely bound or even represent differing specificities contained in the same antigen molecule.[14] Even with such associations to limit possible combinations of antigens within the same bacterium, a great number of serotypes is possible. New serotypes are continuously described, and from time to time the relevant data are aggregated.[63] Combinations of antigens, *e.g.*, serotypes, can be produced in the laboratory (see below), and their natural occurrence is perhaps a consequence of conjugation and transduction as well as loss variations. In any case, the relative fluidity of antigenic structures leads to a certain skepticism as to the status of serotypes in classification (see below) although they are extremely useful in practice.[82]

Such antigenic structures have an illusory precision, illusory in that other antigenic components occur but are neglected for practical purposes, and also because a single antigen, as designated by a symbol, may in fact be a complex in that there are small variations in it as it occurs in different microorganisms. Such differences, as well as similarities, are demonstrable in O antigen having polysaccharide specificities as chemical differences,[77, 80] and also in at least some of the flagellar antigens.[70]

Salmonella typing. Partial or complete identification of such serotypes is carried out by the use of antiserums containing appropriate antibody; monospecific antiserums may be prepared by absorption to contain antibody to a single antigenic component when necessary. The serological identification of Salmonella strains in this way is known as Salmonella typing.

The somatic antigen provides the basis for primary separation of these serotypes into group. There are thirteen such groups in all, the first twelve made up of serotypes having common and identifying O antigens, and the last those serotypes which do not fit in elsewhere. These groups are:

Group A	I, **II**, XII
Group B	(I), **IV**, (X), XII
Group C_1	VI, **VII**
Group C_2	VI, **VIII**
Group D	(I), **IX**, XII
Group E_1	**III**, X
Group E_2	**III**, X
Group E_3	**III**, XIX
Group F	XI
Group G	(I), XIII, XXIII
Group H	(I), VI, XIV, XXV
Group I	XVI

About 98 per cent of Salmonella strains isolated fall into the first eight groups, A to E_3 inclusive, and the identifying antigens of these groups are shown in boldface type above, while those antigens which occur in some but not all serotypes or strains of the group are shown in parentheses.

It is, then, a relatively simple matter to assign a strain to one or another of these groups by the use of appropriate antiserums, and identification may be carried considerably further with rela-

Salmonella Typing Serums

O ANTISERUMS			H ANTISERUMS	
Group	Antiserum	Antigen	Antiserum	Antigen
A	I, II, XII	*Sal. paratyphi A*	a	*Sal. paratyphi A*
B	IV, V, XII	*Sal. paratyphi B*	b	*Sal. paratyphi B*, phase 1
C_1	VI, VII	*Sal. thompson*	c	*Sal. cholerae-suis*, phase 1
C_2	VIII	*Sal. virginia*	d	*Sal. typhi*
D	IX, XII	*Sal. gallinarum*	i	*Sal. typhimurium*, phase 1
E	III, X, XV	*Sal. anatum* and *Sal. newington*	1, 2, 3, 5	*Sal. thompson*, phase 2 and *Sal. newport*, phase 2

tively few additional antiserums to the H antigens. The antigens used to prepare common typing antiserums are shown in the accompanying table. Complete analysis of the antigenic structure of a strain, or identification of the small portion of isolates falling outside these common somatic groups, becomes a specialized procedure.

Classification. The Salmonella group is relatively homogeneous in that these bacteria resemble one another more closely than they resemble other enteric bacilli, although the Arizona and Bethesda-Ballerup groups of paracolon bacilli are "Salmonella-like," and provide connecting links between Salmonella and the coliform bacilli. It is little more than a matter of arbitrary definition as to whether or not the Salmonella group should be made larger and more complex by the inclusion within it of, for example, the Arizona group of paracolon bacilli.

Nomenclature within the group is another matter. Certain well-established types, differentiated by physiological methods prior to the application of antigenic analysis, such as those for which representative antigenic formulas are given, retain names such as *Sal. typhi* and *Sal. enteritidis.* As new and additional serotypes have been described, it has been customary to give them place names and implied species status, *viz.*, *Sal. newport, Sal. montevideo, Sal. panama,* etc. An alternative to the use of a great many such names has been to give these but a single name, such as *Sal enterica,* further identified by antigenic group or formula. This has not met with general acceptance, and the multiplicity of serotypes continue to be known by a multiplicity of names. As indicated earlier (Chap. 20), the use of such names is not to be taken as indicating that these serotypes are species in the Linnaean sense.

Variation. Possibly because the antigenic structure of the Salmonella group is known in considerable detail, three general types of variation may be differentiated. One of these is a type of fluctuating, completely reversible immunological variation known as phase variation, the second induced variation, and the last S-R dissociation known to occur in practically all bacteria.

Phase variation. The H antigens of certain Salmonella types can be separated into relatively stable components. This may be shown very simply by plating out such a type and carrying out slide agglutination of individual colonies with monospecific antiserum. About half the colonies will be found to contain one kind of antigen and about half the other. Apparently the individual bacterial cells do not contain both kinds of H antigen but are of two kinds in this respect. This immunological character breeds true to only a limited extent, for if a colony is picked and subcultured in broth, platings of successive broth cultures will show a rapid reversion to the 50:50 ratio within a very few transfers. This is considered to be a mutation–back mutation process occurring at a relatively high (of the order of 10^{-3} to 10^{-4}) rate.[54, 79] This type of immunological variation is called *phase variation* and the transitory immunological types were originally

called the *specific phase, i.e.,* that characterized by the presence of specific flagellar antigens, and the *nonspecific phase, i.e.,* that in which the nonspecific antigens occurred. Phase variation in one direction only, giving rise to a monophasic strain or mutant, has been observed.[83] This division is not as sharp as once thought, however, and now these phases are commonly referred to as *phase 1* and *phase 2* respectively. The Salmonella types that exist in two immunological phases are termed *diphasic,* while those that exist in only one phase, which may be either phase 1 or phase 2, are called *monophasic.* The presence of three phases appears to occur occasionally.[55]

Types of Phase Variation. Phase variation is somewhat more complex than this, however, and three types have been described. The first is the specific-nonspecific phase variation in which a specific antigen ordinarily occurring in phase 1 is linked with the nonspecific antigens of phase 2. The second is the so-called α-β phase variation in which an antigen occurring in phase 1 is linked with the antigens e, n, +, in phase 2. Five types of α-β phase variation have been reported. Lastly, there is a type of phase variation which has not been named in which the antigens of both phases are commonly found in phase 1.

O antigen variation. It is not clear whether this type of immunological variation occurs generally with respect to the somatic antigens. A similar variation, known as *form variation,* does occur, however. Of the three components of XII, designated XII_1, XII_2, and XII_3, XII_2 varies in that it is either strongly or weakly developed.[25, 46] Antigen VI is likewise subdivided into VI_1 and VI_2 and form variation occurs in the former.[21]

VW Variation. Variation in the Vi antigen content of the typhoid bacillus occurs as a tendency of freshly isolated, Vi-containing strains to lose Vi antigen within a few transplants on culture mediums. Vi-containing strains which presumably contain a maximum amount of this antigen are inagglutinable in O antiserum, the Vi antigen apparently having a masking effect, and are known as V strains. On culture, the V form becomes O-agglutinable, but still contains Vi antigen and absorbs Vi antibody from serum, and is called a VW strain. The VW form then loses its Vi antigen entirely and becomes the W form. The Vi antigen is apparently independent of the other antigenic components of the typhoid bacillus, or those of other bacteria in which it occurs, and its presence or absence is unrelated to the nature of the O and H antigenic complexes.

It is to be emphasized that the immunological changes associated with phase variation are, so far as is known, normal, the bacterial strain seemingly existing in a sort of immunological equilibrium. These variations and the complete but temporary loss of flagellar antigen by cultivation on phenol agar noted above apparently bear no relation to the S-R dissociation, though, as indicated, they may be induced by a method which will induce dissociation, *i.e.,* cultivation in specific antiserum.

Induced variation. Antigenic changes may be produced in the H antigens of a number of Salmonella species by cultivation in the presence of appropriate antiserums. It has been shown that *Sal. paratyphi A,* a monophasic type stable in phase 1, can be induced to form phase 2 antigens by cultivation in antiserum specific to the phase 1 antigens. Conversely, diphasic types can be stabilized in one phase by cultivation in the presence of antiserum to the antigens of the other phase. By this means Salmonella "species" may be transformed, for example, *Sal. simsbury* into *Sal. senftenberg.*[28] Furthermore, "new" species may be created from monophasic types by suppression of flagellar antigen through cultivation in the presence of monospecific antiserum, with the appearance of a hitherto unknown flagellar phase. A number of such induced antigens have subsequently been found to occur naturally.[18]

O antigen specificity is altered in the S-R dissociation as indicated below, but this is presumably unrelated to changes

analogous to induced changes in H antigen that have been produced by cultivation in the presence of antiserum in semisolid mediums. By this means changes have been induced in O antigen within groups only; *Sal. anatum* has been changed to *Sal. newington,* and the change reversed.

Dissociation. S-R dissociation, similar in all respects to that known in other bacteria, occurs in cultures of these bacilli. The dissociation from smooth to rough is manifested as an alteration in colonial morphology and loss of virulence. The change is reflected immunologically as a loss of specificity of the somatic antigens, *i.e.,* the rough forms remain motile. The specificity of these antigens is apparently determined by a polysaccharide haptene, and with the disappearance of the haptene the bacteria acquire a new and common immunological character in the somatic antigens, while the flagellar antigens remain unchanged. A mucoid or M phase in colonial morphology has been reported by some workers which is said to be associated with the development of a new immunological specificity.

Phage Typing.[35] Salmonella strains of the same serotype, and indistinguishable by serological, protective or biochemical tests, may be subdivided into phage types on the basis of their susceptibility to lysis by different races of bacteriophage. Such differentiation is extremely useful for epidemiological purposes when the primary source of infection is the human carrier.

Phage typing of Vi-containing strains of *Sal. typhi* is applied on an international scale, and *Sal. paratyphi A* and *Sal. paratyphi B* are also typed but to a lesser extent. The phage types of the typhoid bacillus are arbitrarily designated as type A, B, etc., with some subtypes, to type T, and there are nine additional types which are numbered or designated with a name, to give a total of thirty-three such types. Type E_1 is the most common all over the world, with type A a close second. In this country the commonest is type E_1, followed in frequency by type C, type A, and type F_1 in that order, but about 10 per cent of strains cannot be typed.

The predominance of one or another of the phage types of *Sal. typhi* may characterize a geographical region. In the Western hemisphere, types C, E_1, D_1, and A occur in that order in Canada, and types A, T, and E_1 in Venezuela. In England the most frequently occurring types are E_1, A, C, and D_1; in Denmark A, F_1, E_1, and C; in Portugal B_3, A, E_1, T, and D; etc. Other phage types are discontinuously distributed, *viz.,* type M is found in Asia, in fact is commonest in Vietnam, and otherwise is found in Iran, France, and Poland.

There are four phage types of *Sal. paratyphi A* and ten types of *Sal. paratyphi B*[8, 10, 62, 69, 74] in both series designated by numbers. These have been less extensively studied than the phage types of *Sal. typhi,* and in both groups type 1 appears to be the commonest wherever studies have been made. Other Salmonella serotypes have not been studied in any detail with respect to phage types, except *Sal. typhimurium*[12, 36] of which some 80 phage types are distinguishable.

Ecology. As noted earlier, bacteria of the Salmonella group all appear to be obligate parasites, and they are not found apart from animal hosts. *Sal. typhi, Sal. paratyphi A,* and usually but not invariably *Sal. paratyphi B* are restricted to man where they cause disease or persist in the carrier state. The remainder of this large group are parasites of lower animals, especially rodents and birds, although they are found occasionally in reptiles. The natural host of *Sal. enteritidis,* for example, seems to be the rat, and that of *Sal. typhimurium,* the so-called mouse typhoid bacillus, the mouse. Fowl are commonly infected, including chickens, turkeys, and ducks, and may constitute the largest reservoir of human infection; of other domestic animals, the pig is most commonly infected, especially with varieties of *Sal. cholerae-suis,* which is not, however, the etiological agent of hog cholera, a virus disease. Of a series of 2520 isolates[24] exclusive of *Sal. pullorum* and *Sal. gallinarum,* the causative agents of bacillary white diar-

rhea of chicks, 1258 were isolated from fowls, 532 from man, 475 from swine, 90 from rodents, 88 from carnivores, 53 from horses and 20 from ruminants. These proportions reflect the kind of specimens examined and hardly constitute a random sample.

The isolates from human sources in this series included forty-one types of Salmonella; of those occurring most frequently, 132 were *Sal. paratyphi B,* 60 were *Sal. typhimurium,* 53 were *Sal. newport,* 37 were *Sal. newington,* 28 were *Sal. cholerae-suis* var. *Kunzendorf,* 27 were *Sal. panama,* and 24 were *Sal. montevideo.* These proportions are probably reasonably representative, but one type or another may predominate in man in a given locality for a time and then, unaccountably, shift. For a period of several years, for example, *Sal. montevideo* was found in the great majority of specimens examined at the University of Chicago Clinics.

The presence of Salmonella is frequently associated with disease, both in man and in lower animals, and epidemics of Salmonella infection may occur in flocks of turkeys and similar situations. The infection may also be symptomless and the host act as a carrier, but the carrier state is usually not chronic except in the case of chronic human carriers of *Sal. typhi* where the gallbladder or urinary bladder is infected, apparently permanently.

Bacteriological Diagnosis of Salmonella Infection.[26, 45] The differentiation of the paratyphoid fevers from typhoid fever and the determination of the etiology of gastroenteritis caused by Salmonella is necessarily dependent on the isolation and identification of the causative microorganism. For isolation both enrichment culture and direct plating should be used; enrichment broth and differential selective agar plates should be inoculated simultaneously and, if the latter are negative, fresh plates can be inoculated from the enrichment culture. It is commonly observed that no single agar medium suffices, for with very few bacteria cultures may be isolated on one medium but not the others; at least two, and better three, kinds of differential agar should be used.

Two enrichment mediums are commonly used. Selenite-F broth contains 0.4 per cent sodium acid selenite, which is toxic for all gram-negative bacteria but is detoxified sufficiently in the presence of 1 per cent sodium phosphate to permit rapid growth of enteric pathogens while temporarily (8 to 12 hours) inhibiting *E. coli.*[75] Tetrathionate broth contains thiosulfate, tetrathionate, and iodide, the tetrathionate being formed by oxidation of the thiosulfate with iodine. The differential selective agars used contain lactose and an indicator (often neutral red), together with bile or bile salts.[43] Of these the most commonly used are desoxycholate-citrate agar (D–C), Shigella-Salmonella agar (S–S), and MacConkey agar. On these mediums the non-lactose-fermenting bacteria form colorless opaque or translucent colonies which are readily differentiated from the red colonies of the lactose-fermenters.

The Wilson-Blair bismuth-sulfite medium is especially useful for the isolation of *Sal. typhi.* The colonies appear black, as do those of *Sal. paratyphi B* and *Sal. enteritidis.* The medium is strongly inhibitory and must be inoculated very heavily; consequently the colonies may not be pure cultures.

Typical colonies are picked and subcultured in Kligler's iron agar or triple sugar iron agar; these mediums indicate acid or acid and gas formation from the sugars present and also the formation of hydrogen sulfide. After 24 hours' incubation, the growth is subcultured in urea medium, citrate medium, motility medium, gelatin, and lactose, sucrose, and mannitol broths. Lactose broth cultures should be retained for not less than 2 weeks to eliminate slow lactose fermentation. A positive lactose (and/or sucrose) fermentation, hydrolysis of urea, or indol formation eliminates Salmonella.

Further identification is serological,[23] first with polyvalent antiserum which does not, however, eliminate certain of the dysentery bacilli (Flexner types 1 and 2) or serologically related paracolon

bacilli of the Arizona group. Serological identification is made more precise by typing with monospecific serums as indicated earlier, either approximately or in detail. The usual technique is a rapid slide agglutination in which a drop of relatively heavy (milky) suspension of the bacteria is placed on a slide and mixed with a drop of antiserum, which may be appropriately diluted to eliminate cross-reactions in some cases. Within a few minutes agglutination occurs, and the milky suspension appears to become curdled. This is most readily observed under low magnification, such as with a hand lens or a dissecting microscope. It is essential that a saline control be included, for rough forms agglutinate spontaneously in salt solution.

Pathogenicity for Lower Animals. Salmonella infection of rodents is quite common; *Sal. typhimurium* and *Sal. enteritidis* cause infections of rats and mice, and these animals may become healthy carriers of the bacilli, a point of importance in connection with the epidemiology of food-poisoning outbreaks. *Sal. typhimurium* infection is by far the most common in the United States, and *Sal. enteritidis* less so than generally supposed. Preparations of "rat virus" or "Ratin" consist of these bacteria and are supposed to initiate an epidemic of disease in the rat population and hence destroy it. The use of such preparations is to be condemned, for not all the rats are killed and many of the survivors become healthy carriers; there is, in addition, the danger involved in leaving preparations of virulent bacilli about the home.

Contrary to what had been previously supposed, the dog may be infected with Salmonellas; as high as 15 per cent incidence has been observed. While it seems unlikely that the dog is an important reservoir of human infection, it has been found to transmit infection to man.[13]

Salmonella infection of the horse is also quite common. Infectious abortion of mares is caused by a specific microorganism, *Sal. abortus equi,* which has not been found in other animals. Man is only rarely infected. *Sal. typhimurium* has also occasionally been reported in horses. Abortion in sheep has been attributed to a member of the Salmonella group, *Sal. abortus ovis.* Salmonella is occasionally observed in a variety of other animals.

Birds are quite commonly infected with members of the Salmonella group. Epidemics due to *Sal. typhimurium* sometimes cause great destruction among canaries and other songbirds. Salmonella infections in turkeys occur and may be of such magnitude as to assume both economic and public health importance.[1] Two barnyard diseases of great economic importance are due to specific Salmonella types: the bacillary white diarrhea of chicks caused by *Sal. pullorum* and fowl typhoid caused by *Sal. gallinarum. Sal. pullorum* may survive in the ovaries of fowls that recover from infection; diseased chicks may develop from the infected ova and communicate the disease to initially healthy members of the flock. Rare cases of human infection with *Sal. pullorum* have been reported, and it has been associated wtih epidemic foodborne gastroenteritis in some instances.

The typhoid bacillus. The typhoid bacillus is, in contrast to most members of the Salmonella group, almost completely nonpathogenic for lower animals, but Koch's third postulate, the production of the disease by microorganisms in culture, has been adequately fulfilled by accidental infections in the laboratory.[67] In addition, typhoid fever, and a subclinical infection, with bacteremia and occasional infection of the gallbladder to produce a chronic carrier state, has been produced in chimpanzees by oral inoculation.[20] The lethal dose for the mouse by the intraperitoneal route is of the order of many millions of bacilli and approaches the toxicity of the killed bacteria. Virulence for the mouse may be greatly increased by suspension of the bacilli in 5 per cent mucin, and the LD_{50} by the intraperitoneal route may be on the order of 10^3 bacteria. The disease produced is a fulminating bacteremia, usually resulting in death within 48 hours. This kind of experimental infection has had wide application in assay of the immunogenic potency of vaccines

and study of the relative importance to immunity of the Vi antigen and various fractions of the O antigenic complex.

A fatal infection with relatively small numbers of bacilli may be produced in the mouse by intracerebral inoculation.[50, 51] The infection disseminates in the central nervous system to produce an acute purulent meningoencephalitis with secondary edema and pyrocephalus. Guinea pigs may be infected by direct inoculation of the gallbladder, and some animals so infected may become chronic carriers.[44] This kind of infection, first described by Bingel with dysentery bacilli, has been used as a criterion of pathogenicity for a number of the Enterobacteraceae.[78]

SALMONELLA INFECTIONS

Salmonella infection in man is almost always acquired by ingestion of the microorganisms, usually as contamination of food, milk, or water. Occasionally double infections occur, *i.e.*, simultaneous infections with more than one kind of Salmonella,[72] but these are not common. Two kinds of disease occur, one an acute gastroenteritis characterized by vomiting and diarrhea of which a small portion of patients become septicemic, and the bacteria are found in the blood in relatively large numbers. The other is the typhoidal or continued fever type of disease in which bacteremia occurs in the initial stage of disease, and the symptoms are more general in nature. *Sal. typhi, Sal. paratyphi A,* usually *Sal. paratyphi B,* occasionally *Sal. paratyphi C* and related forms, and exceptionally almost any other Salmonella serotype, are responsible for the continued fevers. Acute gastroenteritis, on the other hand, is seldom if ever due to infection with *Sal. typhi* or *Sal. paratyphi A,* and a minor portion of cases of infection with *Sal. paratyphi B* are of this kind, but the great majority of cases of gastroenteritis of Salmonella etiology are due to infection with the multiplicity of other Salmonella serotypes, largely *Sal. typhimurium, Sal. enteritidis, Sal. cholerae-suis,* etc. While, then, no sharp distinction can be made between the etiological agents of these two kinds of diseases, there is a general association.

In addition to these main types of disease, a wide variety of diseases may occasionally be associated with Salmonella infection.[9] These microorganisms all have a predilection for the bone marrow, and bone infections are not uncommon, together with other localized infections, infections of the central nervous system, etc. A carrier state, usually transitory except in the case of chronic carriers of *Sal. typhi* (see below), occurs in man with discharge of the microorganisms in the feces. Infection may be acquired from human carriers, and such carriers should not, for example, be food handlers, but probably most human infections are from lower animals, *viz.*, contamination of food with rodent feces, contamination of bulk and dried eggs by infected fowl, etc.

SALMONELLA GASTROENTERITIS

Gastroenteritis of Salmonella etiology is characterized by a short incubation period, as little as 12 hours in some cases, acute vomiting and diarrhea with slight rise in temperature, and rapid recovery, usually within a few days. Occasionally this kind of disease develops into a more serious septicemic condition, and there is a greater tendency to do so when the infectious agent is *Sal. cholerae-suis* and related forms of the "para C group." It is most often acquired by ingestion of contaminated food, and is called Salmonella food poisoning, but is an infection rather than an intoxication as in staphylococcal food poisoning. Salmonella food poisoning seems to be relatively more common than staphylococcal food poisoning in England, and the re-

verse in this country, but it is not clear that there is a real difference.[16, 17] No doubt some, and possibly many, of the descriptions of indigenous cholera and cholera nostra in earlier writings refer to this kind of disease.

The general behavior of Salmonella infection is illustrated in a study of 7779 strains of Salmonella isolated between 1937 and 1955.[66] In this group, gastroenteritis occurred in 68 per cent of the cases and was appreciably more severe in infants and persons over 50, and a typhoidal or septicemic condition occurred in 8.8 per cent. The over-all case fatality rate was 4.1 per cent, with more than half the fatalities occurring in infections with *Sal. typhimurium* and *Sal. cholerae-suis;* in the latter infection, which tended to be septicemic, the case fatality rate was 20.3 per cent. In *Sal. enteritidis* infections, the case fatality rate was 5.8 per cent for all ages, but 15 per cent in those over 50.

Salmonella Typhimurium (*Salmonella aertrycke, Bacterium aertrycke, Bacterium typhimurium*). This bacterium, most commonly isolated in food poisoning outbreaks in the United States and in Great Britain, closely resembles *Sal. paratyphi B* in its cultural characteristics but can be distinguished by its ability to produce acid in tartrate medium. Before differential tests were satisfactorily worked out, *Sal. paratyphi B* and *Sal. typhimurium* were commonly confused and both termed "para B." *Sal. typhimurium* is commonly found in a variety of infections in laboratory and domestic animals and in birds, and the "*B. pestis caviae*" of some writers and Nocard's "*B. psittacosis*" are, in fact, *Sal. typhimurium.* Most of the laboratory stock cultures labeled "Danysz virus" or "bacillus of mouse typhoid" are of the typhimurium type, but some are *Sal. enteritidis.*

Salmonella Enteritidis (*Bacterium enteritidis*). Although found frequently in food-poisoning outbreaks, this bacterium is less common than *Sal. typhimurium.* It closely resembles *Sal. typhimurium* culturally but is said by some to differ in that it does not ferment inositol while *typhimurium* does. The inositol fermentation is, however, frequently not clear-cut; a lowering of pH may be noted but sometimes not to a sufficient degree to warrant calling the fermentation positive. In the writer's experience, the inositol fermentation is variable in both species. The two may be separated by serological means.

Salmonella Cholerae-suis (*Bacterium suipestifer, Bacterium choleraesuis,* the hog cholera bacillus, American suipestifer). This Salmonella is a member of a group of closely related bacteria, called the *suipestifer group,* which also contains *Sal. paratyphi C* or Eastern type, as noted above, *Sal. cholerae-suis* var. *Kunzendorf* or European type, and the Glässer-Voldagsen type, which is comprised of two species, *Sal. typhi-suis* and *Sal. typhi-suis* var. *Voldagsen.* These species may be differentiated from one another by a combination of cultural and serological methods. *Sal. cholerae-suis* formerly predominated in the United States, but now the Kunzendorf variety is found more often. This species has been implicated in outbreaks of paratyphoid gastroenteritis, although to a much lesser extent than either *Sal. typhimurium* or *Sal. enteritidis. Sal. typhi-suis* appears to be an animal (pig) pathogen, but several cases of human infection with the Voldagsen variety have occurred in the United States. The case fatality rate in *choleraesuis* infections is 20 to 25 per cent in contrast to 5 per cent or less in infections with other Salmonellas.

Other Salmonella species are less commonly implicated in human infections. *Sal. thompson* and *Sal. newport,* both related to the suipestifer group immunologically, have been observed a few times and other species but once; in fact, a number of the new Salmonella species described in recent years have been isolated from food implicated in outbreaks of enteritis.

TYPHOID AND PARATYPHOID FEVERS

Salmonella infection resulting in the continued fever kind of disease is charac-

teristically typhoid fever, caused by *Sal. typhi*. As indicated above, the infectious nature of typhoid fever was suspected by the middle of the last century, and the causative agent was among the earlier of the pathogenic bacteria to be described. Within a few years paratyphoid fever was differentiated. Achard and Bensaude isolated a microorganism similar to, but not identical with, the typhoid bacillus from typhoid-like disease in 1896, and 2 years later Gwyn isolated a microorganism closely resembling *Sal. enteritidis* under similar circumstances. Since that time a number of investigators in different parts of the world have isolated similar bacteria from the blood of patients suffering from a disease that, so far as clinical symptoms are concerned, is substantially identical with typhoid fever.

Paratyphoid Fever. Many cases of paratyphoid infection show a tendency to run a relatively mild course and are marked by a sudden onset with chills, but are otherwise very similar to infections with the typhoid bacillus. A certain proportion of the negative agglutination tests in apparent typhoid fever are probably a result of paratyphoid infection. The only method by which typhoid and paratyphoid fever can be distinguished is isolation and identification of the causal microorganism.

Many scattered cases of paratyphoid fever have been observed, and a number of more or less extensive epidemics have been reported as due to milk and other foods, to contact with human carriers, to sewage-polluted water and similar factors. Infection is probably most often acquired from carriers; the bacilli may be excreted for some weeks by convalescents (Fig. 107), and subclinical and undiagnosed cases are similarly a source of infection.[41] In general, the mode of dissemination of paratyphoid fever is practically identical with that of typhoid fever.

The frequency of the paratyphoid fevers as compared with typhoid fever varies a good deal in different localities, but most hospital records give a ratio of less than 1:10. In some regions the proportion of paratyphoid to typhoid may be as high as 1:4 or even more. During the First World War the proportion of paratyphoid to typhoid reached a high point. In the British armies in France

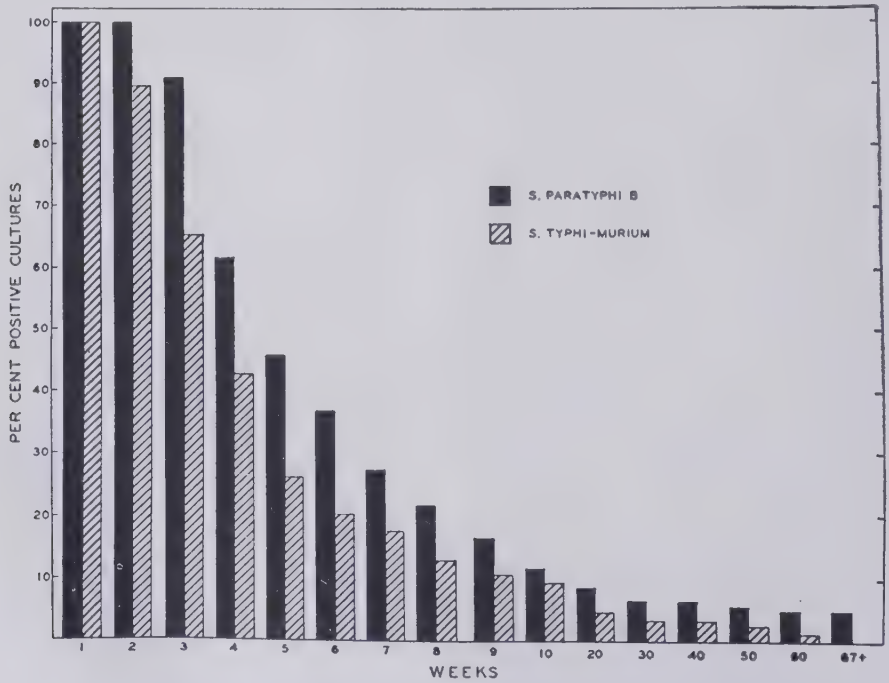


Figure 107. The persistence of Salmonella infection as indicated by positive fecal cultures by weeks. Data on 203 cases of *Salmonella paratyphi B* infection and 239 cases of *Salmonella typhimurium* infection, by Rubenstein, Feemster, and Smith.[65]

during the years 1915 to 1918, the diagnosed paratyphoid fevers outnumbered the typhoid cases 2:1. In civilian populations in most countries paratyphoid fevers probably amount to 5 or 10 up to 50 per cent or more of all *fully diagnosed* enteric cases. Very young individuals appear to be the most susceptible to Salmonella infection, and an unduly large portion, perhaps 20 per cent, of cultures are from young children.

Salmonella paratyphi A (*Bacillus paratyphosus A, Bacterium paratyphosum A, Salmonella paratyphi*). This bacterium differs culturally from most of the other Salmonella species in its inability to ferment xylose, and it is, in addition, serologically distinct. Some outbreaks due to this microorganism have been traced to sewage-contaminated water supplies, others to food contaminated through the agency of human carriers. Paratyphoid fever due to *Sal. paratyphi A* is often very mild, 300 cases occurring in a United States infantry regiment without a single death.

Salmonella paratyphi B (*Bacillus paratyphosus B, Bacterium paratyphosum B, Salmonella schottmülleri*). *Sal. paratyphi B* is readily differentiated from *Sal. paratyphi A*, but has confusing cultural and serological relations with certain Salmonella strains of the food poisoning types. As in the preceding species, the sources and modes of transmission are similar to those of typhoid fever. The source of the bacteria is generally the human carrier, but it has been reported that dogs have been found responsible for small epidemics, and in another instance a cow was responsible for cases of the disease. In the northern United States and in northern Europe infection with this species seems considerably more frequent than with *Sal. paratyphi A*.

Salmonella paratyphi C (*Sal. hirschfeldii*). *Sal. paratyphi C* has been found in enteric fevers in parts of Asia, Africa, and southeastern Europe and has been reported as an important cause of illness and death in British Guiana. Cases of endocarditis have been found to be caused by this and other suipestifer species. Although the type of disease is similar to the other enteric or intestinal forms, little is known about its epidemiology. This species is closely related biologically to the hog cholera or cholerae-suis strains described above.

Other species have been occasionally found: *Sal. barielly*, isolated from cases of mild pyrexia in India; *Sal. enteritidis* var. *moscow*, isolated from cases of paratyphoid fever in Russia and described by Russian bacteriologists as "Paratyphus N_2"; *Sal. sendai*, isolated from cases of paratyphoid fever in Japan; and *Sal. eastbourne*, from a paratyphoid case in Eastbourne, England. In this country *Sal. typhimurium, Sal. saint paul, Sal. oranienburg, Sal. hartford, Sal. sendai, Sal. panama*, and others have been found associated with enteric fevers.

Figure 108. *Salmonella typhi.* Smear from a pure culture, Sommersby strain. Note the variation in size from coccoid to bacillary forms. Fuchsin; × 1050.

Typhoid Fever. Typhoid fever was for long one of the most widespread and important of all bacterial diseases. In the United States in 1900 there were 35,379 reported deaths from this disease, undoubtedly a low figure, and probably some 350,000 cases of typhoid fever in a population of 76,000,000—in the course of a decade perhaps one person in every twenty to twenty-five contracted the disease. The prevalence of typhoid fever has greatly diminished in recent years, and a large part of this decrease has taken place in the large cities. Typhoid still persists, however, and epidemics occur from time to time, particularly in the smaller towns and rural areas. Much the same situation prevails in other countries, such as Great Britain.

The common symptoms of typhoid fever include frontal headache, lack of appetite, nosebleed, the development of rose spots on the abdomen, muscular weakness, and diarrhea. Sometimes considered primarily an intestinal infection, the disease is, in fact, a general invasion of the body, particularly of the lymphatic system. The gastrointestinal tract is the portal of entry of the bacilli into the body; the lymphatic tissues in the intestinal wall are first invaded, and the bacilli spread through the lymphatic system. After considerable multiplication has occurred (incubation period), the bacilli overflow into the blood and bacteriolysis takes place; the endotoxins which are liberated as a result of the destruction of the bacilli produce the symptoms of typhoid fever. Other evidence suggests that the body tissues may be invaded through the tonsils and gastric mucosa, but in any case the end result is the same, and typhoid fever is a general and not a localized infection.

The typhoid bacillus appears in the blood stream early in the disease, *i.e.*, after the onset of symptoms, and may be cultured within the first 10 days in the majority of cases, either from a blood sample or from the clot of samples sent into a laboratory for agglutination tests.[86, 87] The presence of typhoid bacilli in the blood, however, does not constitute septicemia; in fact, probably little or no multiplication takes place. The bacilli are also present in the bone marrow early in the disease, and some have urged culture by sternal puncture to facilitate diagnosis. During and after the second week typhoid bacilli may be found with increasing frequency in the feces, and the proportion of positive blood cultures drops off. The bacilli are also excreted in the urine in perhaps 25 per cent of the cases. They may often be found early in the disease in the rose spots, not in the blood but in the lymphatic spaces.

On autopsy, the intestinal walls are usually found to be extensively ulcerated, Peyer's patches and the solitary glands of the intestine being particularly involved and containing typhoid bacilli. Perforation of the intestinal wall as a consequence of ulceration is a not un-

Figure 109. Colonies of typhoid bacillus on nutrient agar. Note the characteristic "maple-leaf" irregular margin and slightly roughened glistening surface. × 6.

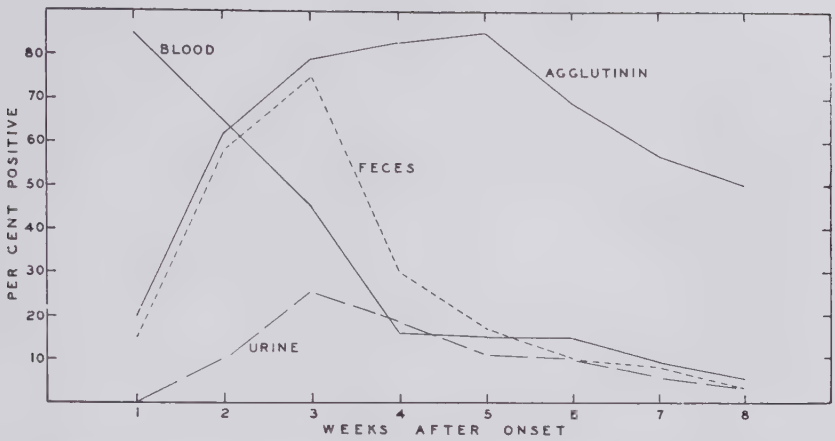


Figure 110. The approximate incidence of positive culture of blood, feces, and urine, and agglutinin response in typhoid fever.

common occurrence. The spleen is enlarged and congested and usually contains large numbers of typhoid bacilli. In both the spleen and the liver the bacilli seen in stained sections occur in groups or masses rather sharply focalized; scattered individuals are not often found.

A variety of complications may occur. Laryngeal ulcer is occasionally observed. The gallbladder is often infected, and cystitis sometimes occurs. Suppurative and inflammatory processes may appear in other parts of the body. The osseous system seems especially open to attack, and affections of the periosteum, the bone marrow, and the joints have been traced to infection with *Sal. typhi.* Osteomyelitis may develop as long as 6 or 7 years after recovery from typhoid fever, indicating that the typhoid bacillus can remain in contact with human tissues for years without losing its virulence. Other parts of the body are more rarely invaded during typhoid fever, but almost any organ can be attacked occasionally. The presence of *Sal. typhi* has been reported in a brain abscess, and the cerebral and meningeal symptoms occurring in many cases of typhoid fever are directly connected with the localization of the bacilli in the meninges; bacilli have been found in spinal fluid obtained by lumbar puncture. Pulmonary involvement may occur also, possibly more often than has been supposed.[61]

Secondary or mixed infections, especially with the pyogenic cocci and the pneumococcus, are not at all uncommon and sometimes result in serious complications. Mixed infections with the tubercle bacillus and the anthrax bacillus have also been observed.

Chemotherapy. The typhoid bacillus is relatively resistant to penicillin *in vitro,* but although it is susceptible under these conditions to the other common antibiotics, chloramphenicol appears to be the only one that is reasonably effective *in vivo.* It is, therefore, the chemotherapeutic agent of choice and has given highly promising results. It has been reported that relapses tend to occur unless the drug is given for 2 to 4 weeks. Some attempts have been made to cure the carrier state by chemotherapy, with encouraging results in some instances but not in others.

Carriers.[56] About one-third of the individuals having typhoid fever discharge bacilli for a period of 3 weeks after the onset of illness and about 10 per cent for 8 to 10 weeks; these are known as convalescent carriers.[68] A certain proportion continue to discharge typhoid bacilli for 6 months or more, and in many cases over a period of several years or throughout the whole of a long life.

The development of the carrier condition is probably dependent upon the invasion of the gallbladder in the case of the fecal carriers and of the urinary bladder in the case of the urinary carriers. Fecal carriers are more common than

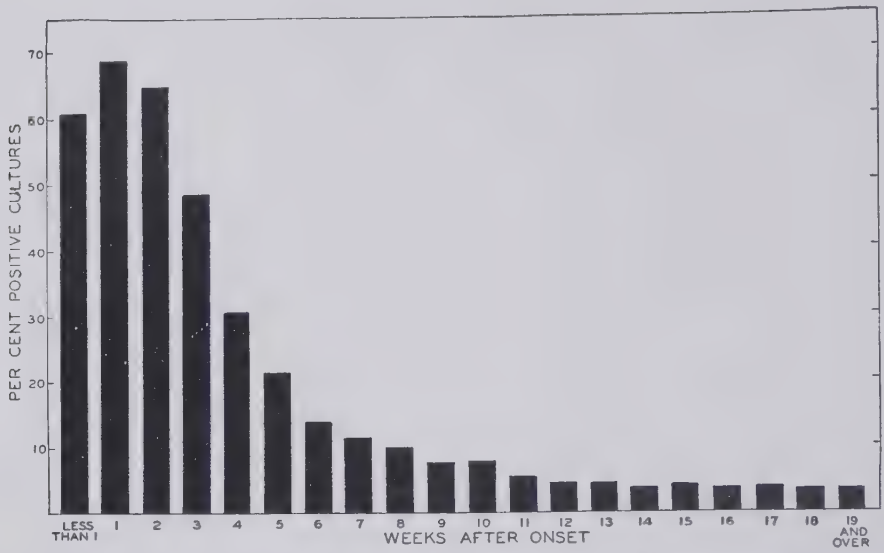


Figure 111. The persistence of typhoid bacillus infection as indicated by percentage of positive fecal cultures by weeks after onset. Data from 374 cases in New York State exclusive of New York City, by Ames and Robins.[2]

urinary carriers, and combined fecal and urinary carriers are relatively uncommon. The development of the urinary carrier state is apparently favored by pathology of the urinary tract produced, for example, by urinary schistosomiasis, and this probably accounts at least in part for the high incidence of urinary carriers in certain areas such as Egypt. Urinary carriers have been found to excrete antibody in the urine.[4] It is not known why women are more commonly carriers than men. In the series studied by Ames and Robins,[2] 2.1 per cent of the males became chronic carriers as compared with 3.8 per cent of the females. Age is a factor also; according to the same workers the percentage of cases becoming carriers was 0.3 in the 0 to 9 and 10 to 19 age groups, but as high as 10.1 in the 50 to 59 age group. The usual estimates for all age groups vary from 0.5 to 11.6 per cent.

Typhoid bacilli need not be excreted continuously; in fact, their intermittent appearance is very common, and weeks may elapse with negative cultures before the bacilli reappear.[64] The necessity for repeated examinations is obvious. The numbers of bacilli may vary widely, from 500,000 to 450,000,000 per gram of feces in one study.[84] A majority of carriers give the Widal reaction, and in most cases the opsonic index is abnormally high. Antibody to Vi antigen is found in the great majority of carriers but is only transitory if present at all in inoculated persons, and the use of the Vi agglutination test for the detection of carriers has given encouraging results.[71]

Attempts to cure typhoid carriers by nonsurgical means, such as chemotherapy, vaccine therapy, or bacteriophage, have not been generally successful, and such procedures are not generally advocated at the present time. Removal of the gallbladder under suitable conditions is often effective in the case of fecal carriers; 70 per cent is the usually accepted figure.

The proportion of typhoid carriers in the general population is not known, owing to obvious practical and technical difficulties. It is probably quite different in different localities and doubtless depends largely upon the prevalence of current and past typhoid infection. By a modified life table procedure Ames and Robins[2] estimated a carrier rate of 42 per 100,000 in New York State. On the basis of reported deaths and assuming a case fatality rate of 10 per cent and a chronic carrier incidence of 2 per cent in survivors, carrier rates have been estimated

as 48 per 100,000 in Massachusetts and 288 in Mississippi.

The number of carriers throughout the United States is probably decreasing since they are no longer produced in such quantities as when typhoid fever was prevalent. By extrapolation Ames and Robins[2] calculated that the 2500 carriers in New York State in 1940 would be reduced to 200 by 1980.

Epidemiology. The typhoid bacillus is a strict parasite found only in man. Outside the human body multiplication, if it occurs at all, is insignificant, and for practical purposes may be neglected as a factor in the dissemination of the disease. As indicated above, the typhoid bacillus leaves the body in the feces or, less commonly, the urine, and enters the body of a new host via the alimentary tract. The epidemiology of typhoid fever, then, is predicated upon the connection between the intestinal tract of the infected person and the mouth of the susceptible, and the factors that determine the spread of this disease are, essentially, those arising as a consequence of the inter-relationships of the individuals or groups of individuals comprising the host population. The extent of the spread of typhoid is dependent upon the nature of the connecting links between individuals, and two epidemiological types of the disease may be distinguished, the one epidemic typhoid, the other endemic, or residual, typhoid.

Epidemic typhoid fever. Extensive outbreaks of typhoid fever necessarily involve a connecting link that is common to a great many people, and by far the most important vectors of this kind are water and milk. As pointed out elsewhere (Chap. 10), water-borne typhoid fever, formerly all too common but by now relatively rare in the larger communities, occurs as a consequence of the contamination of a water supply with infectious fecal material, either as such or in the form of sewage. Water-borne epidemics of typhoid fever occur in the absence of chlorination, filtration, and other purification procedures and may be readily prevented. These epidemics tend to occur in the cold months of the year, particularly in the winter and early spring, and the incidence of the disease is unaffected by age, sex, or economic status.

Milk-borne typhoid fever, at the beginning of the twentieth century second only to water-borne typhoid in extent and importance, follows the route of the milkman and, as might be expected, tends to occur in the lower age groups and in families of higher economic status. The general introduction of the process of pasteurization has practically eliminated milk-borne typhoid fever from the larger urban centers, but epidemics continue to occur from time to time in various parts of the country.

Food-borne typhoid fever may take on epidemic proportions in certain instances. Oysters and other shellfish have come into bad repute in this respect in recent years, for a number of typhoid epidemics in Great Britain and the United States have been found to be due to the eating of oysters grown near sewer outfalls or placed to "fatten" in the polluted waters of estuaries or creeks. Water cress, lettuce, radishes, or any vegetables or fruits which are liable to come in contact with contaminated water or are sprayed with human excrement may give rise to small-scale epidemic typhoid fever.

Endemic typhoid fever. Although epidemic typhoid fever is largely eliminated in a given community through adequate sanitary control of water and milk supplies and such food supplies as are susceptible to the application of effective control measures, the disease remains in an endemic form which is manifested as occasional cases or small groups of cases which appear from time to time. The seasonal incidence is quite different from that of water-borne typhoid; for some years there has been a sharp peak in the summer, but with continued reduction in the incidence of the disease, this has tended to disappear to give a relatively even distribution of cases throughout the year. The source of infection is, of course, the case, frank or ambulatory, or the healthy carrier. Instances of direct contact infection are un-

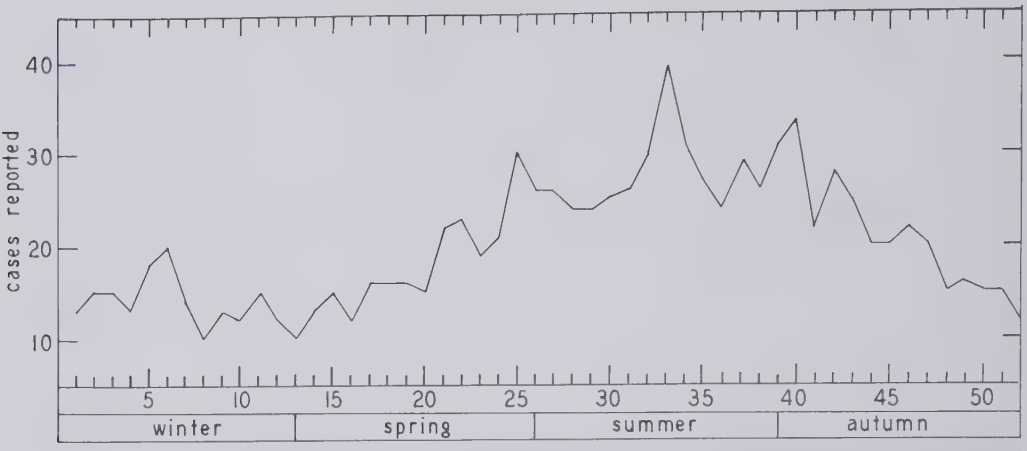


Figure 112. The seasonal incidence of typhoid fever illustrated by the 5-year median number of cases reported weekly, 1956–60. Data from the Morbidity and Mortality Weekly Report, vol. 9, 1961. Communicable Disease Center, Public Health Service, U. S. Department of Health, Education and Welfare.

questionably more common than is generally recognized, and the dissemination of typhoid bacilli from the infected individual to his immediate associates is undoubtedly responsible for the majority of cases of residual typhoid. Carriers are of particular importance in that they constitute semipermanent foci of infection and, when employed as food handlers, may be the cause of small epidemics. The most notorious instance of this kind was that of Mary Mallon, "Typhoid Mary," who was unknowingly the cause of some twenty-six cases of typhoid fever in seven different families. Under special circumstances, such as prevail among troops, contact infection may assume epidemic proportions.

The reduction in the prevalence of typhoid fever in the last few decades is attributable almost entirely to elimination of the great water-borne and milk-borne epidemics. As noted above, epidemics still occur, and their control is a matter of putting into practice existing knowledge. Residual typhoid, however, is much more difficult to control; the detection and supervision of all carriers, or even the elimination of carriers as food handlers, is a practical impossibility. There is reason to believe that with continued control of epidemic typhoid fever, the reduction in the proportion of carriers may well be reflected in a reduced incidence of the disease in the epidemic form.

Immunity. An attack of typhoid fever confers a certain degree of immunity, although instances of two or even more attacks in the same individual are not unknown.[39] Animal experiment has shown that it is possible to obtain a high degree of immunity in rabbits and guinea pigs against intraperitoneal inoculation. Recovery from experimental enteric infection in the chimpanzee is also associated with an immunity to subsequent challenge by the oral route, 18 months after the primary infection.[40] The immunity is associated with the development of humoral antibodies such as agglutinins, precipitins, and the like. Lysin is also produced, and, like the cholera vibrio, the typhoid bacillus undergoes visible dissolution and disintegration in the peritoneal cavity of the immune animal.

In man recovery from typhoid fever is also accompanied by the appearance of demonstrable antibodies. In most instances agglutinins appear during the course of the disease, sometimes as early as the fifth day (over 90 per cent by the fourth week), and their presence is the basis of the Widal test used for diagnostic purposes.

The Widal test. In its original form the Widal test was a slide agglutination

test, and agglutination of typhoid bacilli by patient's serum in a dilution of 1:50 or more was considered positive. The development of knowledge of the antigenic structure of the typhoid and paratyphoid bacilli in recent years has resulted in a somewhat better understanding of the value and limitations of the aglutination test in the diagnosis of typhoid fever. At the present time the test is a macroscopic one and is carried out with both H and O antigens. The interpretation of a single such test must take into consideration ancillary data such as a previous immunization or attack of typhoid fever and the prevalence of endemic typhoid in the general population. It is, therefore, difficult to set arbitrary limits; in most instances an O titer of 1:100 and an H titer of 1:200 may be regarded as significant. A point of some interest is the lack of sharp antigenic specificity of human serums as compared with the specificity of experimentally produced rabbit antiserums.

The interpretation of the Widal test in immunized persons is often difficult, since both H and O agglutinins are formed in response to the vaccine. Titers fall after immunization but may persist at moderate levels for many months. Furthermore, the agglutinin titer may rise in anamnestic response to a febrile condition. The extent to which this occurs is not definitely known, and some data suggest that it is significant while others do not. Such an anamnestic reaction is particularly prone to occur in typhus fever, and typhoid agglutinin titers as high as 1:800 have been observed. There is some evidence that an "agglutinin curve," obtained by periodic agglutinin titrations, has diagnostic value in that it continues to rise in typhoid fever but usually does not do so in the anamnestic reaction.

Prophylactic inoculation.[6, 31] Man may be actively immunized against typhoid fever by the parenteral inoculation of killed typhoid bacilli. Antityphoid vaccination has had its widest application thus far in the protection of soldiers in the field, for, owing to the conditions under which they must live in the field or on the march, the likelihood of typhoid fever is great. Mass immunization has effected a remarkable reduction in the incidence of typhoid fever among these men. For example, in 1898 at the time of the Spanish-American War, 4422 cases of typhoid and 248 deaths occurred in a division of 10,759 men; among 12,-

Figure 113. The prevalence of typhoid and paratyphoid fevers as shown by the mortality rates in the Registration Area. Data from the Morbidity and Mortality Weekly Report, vol. 9, 1961. Communicable Disease Center, Public Health Service, U. S. Department of Health, Education and Welfare.

801 vaccinated men under very similar conditions during summer maneuvers at San Antonio, Texas, in 1911, only one case developed. Similar results have been obtained in the armies of other nations, and the efficacy of typhoid immunization is undoubted. Typhoid vaccination has been compulsory in the United States Army since 1911, and the result has been the practical disappearance of the disease.[11] Military experience does not constitute a controlled experiment; appropriately controlled field tests of sufficient magnitude to have significance are a difficult matter, but those which have been carried out have substantiated the development of a significant immunity by prophylactic inoculation.[15, 19] The immunity so developed is not absolute, of course, and may be broken down by large doses of typhoid bacilli; typhoid fever in immunized personnel of armies is observed from time to time.[3, 81]

The vaccine ordinarily consists of a saline suspension of killed bacteria. The microorganisms are grown on the surface of agar culture mediums, and after 18 hours' incubation are washed off with sterile physiological salt solution and killed by heating to 55° to 56° C. for 1 hour. The suspension is standardized by counting the number of bacteria and then diluting so that 1 ml. contains one billion bacilli.

The vaccine is given in three injections, five days to a week apart, of 0.5 ml. or 500 million bacilli each. As a rule, the reaction following inoculation is not severe, although fever, chills, nausea, and nervous symptoms may be observed. At times it has been found advantageous to immunize simultaneously against the paratyphoid infections as well. Such vaccines, known as TAB vaccines (typhoid, para A, and para B), are standardized to contain one billion typhoid bacilli, 250 million paratyphoid A bacilli, and 250 million paratyphoid B bacilli per ml. Under exceptional circumstances still other microorganisms have been added to typhoid vaccines.

While the effective immunogenic potency of typhoid vaccines can, in the last analysis, be measured only by field trial, protection against the experimental infection of mice with bacilli suspended in mucin has been useful. The active and passive mouse protection tests have been used by workers in the United States Army Medical School in extensive studies directed toward improvement of the immunizing antigen.[57, 73] It is reasonably well established that antibody to the O antigen is protective while that to the H antigen is not,[85] and it is also clear that antibody to Vi antigen is protective against infection with Vi-containing bacteria. In general, then, the vaccine should contain adequate amounts of undenatured O antigen, and its immunizing potency may also be reinforced by the presence of Vi antigen since most strains of typhoid bacilli isolated from human infections contain it. The Army investigations have shown that highly immunogenic strains of the typhoid bacillus are highly virulent and it is concluded that such strains should be used for vaccine preparation. The classic Rawlings strain was found to be inferior as assayed by mouse protection, and a highly virulent, Vi-containing strain, No. 58, has been substituted for it in Army vaccine. Subsequent work has suggested that the vaccine may be further improved by fortification with Vi-containing extracts, but such possibilities are still in an experimental stage. Presumably results based on mouse assay may be applied to man, but as yet there is no definite evidence that vaccine prepared with a Vi strain confers a more effective prophylactic immunity in man than Rawlings strain vaccines. The contribution of Vi antigen to the immunogenic potency of typhoid vaccines is questioned from time to time.[38, 58, 76]

At the present time typhoid vaccines prepared in the United States must conform to an immunogenic potency standard based on the active mouse protection test.

Each of 30 or more mice of any susceptible strain, 6–8 weeks old and weighing 14–16 gms., is given 0.5 ml. of a 1:10 dilution of the vaccine intraperitoneally. Equal numbers of male and female mice should be used in each group. Fourteen days after the injection of vaccine the mice are divided into three groups of not less

Figure 114. *Alcaligenes fecalis.* Smear from a pure culture. Fuchsin; × 1050.

than 10 mice each, one group to receive approximately 100,000, one group approximately 10,000 and the third group approximately 1,000 lethal doses of virulent typhoid bacilli (16–20 hours old) suspended in 5 per cent mucin.[7]

At least 50 per cent of the mice should be protected against not less than 10,000 lethal doses of organisms.

Another type of antigen, consisting of a formalized solution of the cell substance of typhoid bacilli and designated endotoxoid vaccine, has been used with encouraging results,[42] but it is not as yet clear how this antigen compares with whole bacilli.

As indicated above, typhoid vaccines are ordinarily administered parenterally, by subcutaneous injection. It has been contended by some workers that oral administration of the vaccine together with a small quantity of bile, which presumably increases the permeability of the intestinal wall, produces an equally effective immunity. As pointed out in an earlier chapter, oral vaccination is a highly uncertain method of inoculation, having no advantages over parenteral injection. Vaccination by the oral route is seldom practiced.

Passive immunization. The use of antityphoid serum for therapeutic purposes has been considered by a number of workers, but there is still no conclusive evidence as to its value. Whether or not "anti-endotoxic" serums or other typhoid antiserums confer a passive immunity to typhoid infection in man is likewise not established.

ALCALIGENES FECALIS

Alcaligenes fecalis or *Bacterium fecalis alcaligenes* closely resembles the typhoid bacillus morphologically, culturally and even in its growth on Endo, Conradi-Drigalski, and malachite green differential mediums. It has been found in feces and in water. It differs from the typhoid bacillus in the possession of one or more polar, instead of many peritrichous, flagella, and in distinct alkali production in mannitol and litmus milk, and it fails to produce acid from dextrose and other carbohydrates. It has been suggested that *Bact. alcaligenes* is a form of *Bact. fluorescens non-liquefaciens* which has lost the function of pigmentation, and it does have affinities with certain of the plant pathogens and soil bacteria. Its systematic position has been examined critically by Conn who has proposed a new genus, *Agrobacterium,* to include this bacillus together with *Bact. radicicola* and the bacterium of hairy root disease. Other species have been described as occurring in the intestinal tract, *viz., A. metalcaligenes, A. bookeri* and *A. recti,* but these are not ordinarily differentiated from *A. fecalis.* Other varieties, *A. viscosus* and *A. marshallii,* are found in dairy products and produce a slimy alkalinity and ropiness in milk.

While *A. fecalis* is only feebly pathogenic, it has been found in cases of bacteremia, meningitis, infection of the gall bladder, renal tract, eye, lymph nodes, and appendix on occasion.[90] It has also

been found to be the etiological agent of a disease closely resembling "red leg" (of Proteus etiology) in tree frogs.[59]

REFERENCES

1. Abelseth, M. K., and Robertson, H. E.: *Salmonella typhymurium* infection in 1952 turkey flocks—a public health hazard. Canadian J. Pub. Health, 1953, **44:**263–265.
2. Ames, W. R., and Robins, M.: Age and sex as factors in the development of the typhoid carrier state, and a method for estimating carrier prevalence. Amer. J. Pub. Health, 1943, **33:** 221–230.
3. Anderson, E. S., and Richards, H. G. H.: An outbreak of typhoid fever in the Middle East. J. Hyg., 1948, **46:**164–172.
4. Archer, G. T. L., Goffe, A. P., and Ritchie, A.: Studies on urinary carriage of enteric group organisms. J. Roy. Army Med. Corps, 1952, **98:**40–47; 125–131; 189–197; 237–240.
5. Baker, E. E., Whiteside, R. E., Basch, R., and Derow, M. A.: The Vi antigens of the Enterobacteraceae. I. Purification and chemical properties. J. Immunol., 1959, **83:**680–686.
6. Batson, H. C.: Typhoid fever prophylaxis by active immunization. Pub. Health Repts., 1949, Suppl. No. 212.
7. Batson, H. C., Brown, M., and Oberstein, M.: Mouse-protective potency assay of typhoid vaccine. As performed at the Army Medical Service Graduate School. Pub. Health Repts., 1951, **66:**789–805.
8. Bernstein, A.: Laboratory investigations of a carrier of multiple phage types of *Salmonella paratyphi B*. J. Hyg., 1960, **58:**201–206.
9. Black, P. H., Kunz. L. J., and Swartz, M. N.: Salmonellosis—a review of some unusual aspects. New England J. Med., 1960, **262:**811–817; 864–870; 921–927.
10. Brandis, H., and Storch, I.: Über die Lysotypie von Paratyphus B Bakterien mit zusätzlichen Phagen von Scholtens. Zent. f. Bakt., I Abt., Orig., 1957, **169:**358–365.
11. Callender, G. R., and Luippold, G. F.: The effectiveness of typhoid vaccine prepared by the U. S. Army. J. Amer. Med. Assn., 1943, **123:** 319–321.
12. Callow, B. R.: A new phage-typing scheme for *Salmonella typhi-murium*. J. Hyg., 1959, **57:** 346–359.
13. Carter, H. S., and Wier, I. B. L.: A dog as a probable source of human infection with *Salmonella paratyphi-B*. J. Path. Bact., 1952, **64:**230–232.
14. Cohen, H. H.: Mutual linkage between the components in IX and XII in *Salmonella typhi* O-antigen. J. Immunol., 1958, **81:**445–451.
15. Cvjetanovic, B. B.: Field trial of typhoid vaccines. Amer. J. Pub. Health, 1957, **47:**578–581.
16. Dack, G. M.: Food Poisoning. University of Chicago Press. 3rd ed., 1956.
17. Dewberry, E. G.: Food Poisoning: Food-borne Infection and Intoxication. Nature, History and Causation. Measures for Prevention and Control. Leonard Hill, Ltd., London. 4th ed., 1959.
18. Douglas, G. W., McWhorter, A. C., and Edwards, P. R.: Natural occurrence of induced or artificial flagellar antigens in *Salmonella newport*. J. Bact., 1962, **83:**348–350.
19. Edsall, G., Carlson, M. C., Formal, S. B., and Bennenson, A. S.: Laboratory tests of typhoid vaccines used in a controlled field study. Bull. W. H. O., 1959, **20:**1017–1032.
20. Edsall, G., et al.: Studies on infection and immunity in experimental typhoid fever. I. Typhoid fever in chimpanzees orally infected with *Salmonella typhosa*. J. Exptl. Med., 1960, **112:**143–166.
21. Edwards, P. R.: Form variation in group C Salmonella strains. Proc. Soc. Exptl. Biol. Med., 1945, **59:**49–52.
22. Edwards, P. R.: Preparation of antisera for detection of the somatic antigens of Salmonella cultures. Pub. Health Repts., 1951, **66:**837–839.
23. Edwards, P. R.: Simplified serologic identification of Salmonella cultures. Communicable Disease Center, Public Health Service, U. S. Department of Health, Education, and Welfare. 1952.
24. Edwards, P. R., and Bruner, D. W.: The occurrence and distribution of Salmonella types in the United States. J. Infect. Dis., 1943, **72:** 58–67.
25. Edwards, P. R., and Bruner, D. W.: Form variation in *Salmonella pullorum* and its relation to X strains. Cornell Vet., 1946, **36:**318–324.
26. Edwards, P. R., and Ewing, W. H.: A manual for enteric bacteriology. Communicable Disease Center, Public Health Service, U. S. Department of Health, Education, and Welfare. 1951.
27. Edwards, P. R., and Ewing, W. H.: Identification of the Enterobacteraceae. Burgess, Minneapolis, Minnesota. 2nd ed., 1962.
28. Edwards, P. R., Moran, A. B., and Bruner, D. W.: Intertransformability of *Salmonella simsbury* and *Salmonella senftenberg*. Proc. Soc. Exptl. Biol. Med., 1947, **66:**230–232.
29. Ewing, W. H.: Enterobacteraceae. Biochemical methods for group differentiation. Communicable Disease Center, Public Health Service, U. S. Department of Health, Education, and Welfare. 1960.
30. Ewing, W. H., and Edwards, P. R.: The principal divisions and groups of Enterobacteraceae and their differentiation. Internat. Bull. Bact. Nomen. Taxon., 1960, **10:**1–12.
31. Felix, A.: The preparation, testing and standardization of typhoid vaccine. J. Hyg., 1951, **49:** 268–287.
32. Felix, A.: The properties of different Salmonella Vi antigens. J. Hyg., 1952, **50:**515–539.
33. Felix, A.: The Vi antigen of *Salmonella paratyphi A*. J. Hyg., 1952, **50:**540–549.
34. Felix, A.: The Vi antigen of *Salmonella paratyphi B*. J. Hyg., 1952, **50:**550–557.
35. Felix, A.: World survey of typhoid and paratyphoid-B phage types. Bull. W. H. O., 1955, **13:**109–170.
36. Felix, A.: Phage typing of *Salmonella typhimurium:* Its place in epidemiological and epizootiological investigations. J. Gen. Microbiol., 1956, **14:**208–222.
37. Felix, A., and Pitt, R. M.: The pathogenic and immunogenic activities of *Salmonella typhi* in relation to its antigenic constituents. J. Hyg., 1951, **49:**92–110.

38. Fisek, N. H., Gören, S., and Akyay, N.: Mouse challenge with *Salmonella typhosa* T 5501 in testing the potency of typhoid vaccine. Bull. W. H. O., 1959, **20:**1257–1259.
39. Frank, W. P., Bower, A. G., and Chudnoff, J.: Typhoid. Report of a case with three attacks in one year. J. Amer. Med. Assn., 1951, **147:** 1137–1138.
40. Gaines, S., Tully, J. G., and Tigertt, W. D.: Studies on infection and immunity in experimental typhoid fever. II. Susceptibility of recovered animals to re-exposure. J. Exptl. Med., 1960, **112:**1023–1036.
41. George, T. C. R., Harvey, R. W. S., and Thomson, S.: The measurement of the duration of infection in paratyphoid fever. J. Hyg., 1953, **51:**532–540.
42. Grasset, E.: L'endoanatoxine typhoparatyphique dans la prophylaxie des infections typhoidiques. Applications et resultats d'ensemble de quinze ans de vaccination. Rev. d'Immunol., 1951, **15:**1–19.
43. Hajna, A. A., and Damon, S. R.: New enrichment and plating media for the isolation of Salmonella and Shigella organisms. Appl. Microbiol., 1956, **4:**341–345.
44. Henneberg, C.: Meerschweinchen als Typhusbakterienausscheider. Ztschr. f. Hyg. u. Infektionskr., 1953, **136:**383–391.
45. Hormaeche, E., and Peluffo, C. A.: Laboratory diagnosis of Shigella and Salmonella infections. Bull. W. H. O., 1959, **21:**247–277.
46. Kauffmann, F.: A typhoid variant and a new serological variation in the Salmonella group. J. Bact., 1941, **41:**127–140.
47. Kauffmann, F., and Edwards, P. R.: A revised, simplified Kauffmann-White schema. Acta Path. Microbiol. Scand., 1957, **41:**242–246.
48. Landy, M.: Studies on Vi antigen. VI. Immunization of human beings with purified Vi antigen. Amer. J. Hyg., 1954, **60:**52–62.
49. Landy, M.: Studies on Vi antigen. VII. Characteristics of the immune response in the mouse. Amer. J. Hyg., 1957, **65:**81–93.
50. Landy, M., Gaines, S., and Sprinz, H.: Studies on intracerebral typhoid infection in mice. I. Characteristics of the infection. Brit. J. Exptl. Path., 1957, **38:**15–24.
51. Landy, M., Gaines, S., and Sprinz, H.: Studies on intracerebral typhoid infection in mice. II. Immunological factors concerned in protection. Brit. J. Exptl. Path., 1957, **38:**25–34.
52. Landy, M., Johnson, A. G., Webster, M. E., and Sagin, J. F.: Studies on the O antigen of *Salmonella typhosa*. II. Immunological properties of the purified antigen. J. Immunol., 1955, **74:** 466–478.
53. Landy, M., Webster, M. E., and Sagin, J. F.: Studies on Vi antigen. V. Comparison of the immunological properties of Vi antigens derived from V form Enterobacteraceae. J. Immunol., 1954, **73:**23–28.
54. Lederberg, J., and Iino, T.: Phase variation in Salmonella. Genetics, 1956, **41:**743–757.
55. Le Minor, L., and Edwards, P. R.: Présence de trois phases de l'antigène flagellaire chez des bactéries du groupe Salmonella. Ann. Inst. Pasteur, 1960, **99:**469–474.
56. Littman, A., et al.: The chronic typhoid carrier. I. The natural course of the carrier state. Amer. J. Pub. Health, 1948, **38:**1675–1679.
57. Longfellow, D., and Luippold, G. F.: Typhoid vaccine studies. VII. Typhoid-paratyphoid vaccine. Amer. J. Pub. Health, 1943, **33:**561–568.
58. Mandel, A. D., Baron, L. S., and Buckler, C. E.: Role of Vi antigen in *Salmonella paratyphi C* infections. Proc. Soc. Exptl. Biol. Med., 1959, **100:**653–656.
59. Miles, E. M.: Red-leg in tree frogs caused by *Bacterium alkaligenes*. J. Gen. Microbiol., 1950, **4:**434–436.
60. National Library of Medicine: Salmonella; Salmonella infections. Bibliography of literature 1955–April 1960. Public Health Service, U. S. Department of Health, Education and Welfare. 1960.
61. Neva, F. A.: Pulmonary involvement in typhoid and paratyphoid fevers. Ann. Intern. Med., 1950, **33:**83–99.
62. Pöhn, H. P., Rische, H., Brandis, H., and Schneider, H.: Zur Lysotypie von Typhus- und Paratyphus-B-Erregern in Deutschland in den Jahren 1953–1957. Arch. f. Hyg. u. Bakt., 1960, **144:**505–518.
63. Report: Enterobacteraceae Subcommittee of the Nomenclature Committee. Supplement to the sixth report on the Salmonella group. Internat. Bull. Bact. Nomen. Taxon., 1958, **8:**79–91.
64. Report: The detection of the typhoid carrier state. Report of the P.H.L.S. working party on the bacteriological examination of waterworks employees. J. Hyg., 1961, **59:**231–247.
65. Rubenstein, A. D., Feemster, R. F., and Smith, H. M.: Salmonellosis as a public health problem in wartime. Amer. J. Pub. Health, 1944, **34:**841–853.
66. Saphra, I., and Winter, J. W.: Clinical manifestations of salmonellosis in man. An evaluation of 7779 human infections identified at the New York Salmonella Center. New England J. Med., 1957, **256:**1128–1134.
67. Schäfer, W.: Ueber Laboratoriumsinfektionin, insbesondere mit Typhusbazillen. Arch. f. Hyg. u. Bakt., 1950, **132:**15–32.
68. Schäfer, W.: Über die Dauer der Infektiosität von Salmonellosen. Zent. f. Bakt., I Abt., Orig., 1958, **172:**272–281.
69. Scholtens, R. T.: De faagtypering van *Salmonella paratyphi B* in Nederland. Nederl. Tijdschr. v. Geneesk., 1960, **104:**1029–1038.
70. Schrire, L.: The a-z-z_{35} group of Salmonella antigens. Nature, 1960, **186:**569–570.
71. Schubert, J. H., Edwards, P. R., and Ramsey, C. H.: Detection of typhoid carriers by agglutination tests. J. Bact., 1959, **77:**648–654.
72. Shaw, A. B., and Mackay, H. A. F.: Double enteric infection ("la fièvre typhoide intriquée"). An account of an epidemic. J. Hyg., 1951, **49:**299–314.
73. Siler, J. F., et al.: Immunization to Typhoid Fever. American Journal of Hygiene Monograph Series, No. 17. Johns Hopkins University Press, 1941.
74. Sloan, R. S., Wilson, H. D., and Wright, H. A.: The detection of a carrier of multiple phage types of *Salmonella paratyphi B*. J. Hyg., 1960, **58:**193–200.
75. Smith, H. G.: On the nature of the selective action of selenite broth. J. Gen. Microbiol., 1959, **21:**61–71.
76. Standfast, A. F. B.: Experiments with Vi-negative strains of *Salmonella typhi*. Bull. W. H. O., 1960, **23:**47–52.
77. Staub, A. M., Tinelli, R., Lüderitz, O., and Westphal, O.: Étude immunochimique sur les Sal-

monella. V. Rôle de quelques sucres, et en particulier des 3–6 didésoxyhexoses, dans la spécificité des antigènes O du tableau de Kauffmann-White. Ann. Inst. Pasteur, 1959, **96:**303–332.

78. Stenzel, W.: Untersuchungen über die Harnblasenpathogenität einiger Enterobacteraceen-Gruppen im Bingelschen Versuch. Ztsch. f. Hyg. u. Infectionskr., 1960, **147:**123–132.
79. Stocker, B. A. D.: Measurements of rate of mutation of flagellar antigenic phase in *Salmonella typhi-murium*. J. Hyg., 1949, **47:**398–413.
80. Stocker, B. A. D., Staub, A. M., Tinelli, R., and Kopacka, B.: Étude immunochimique sur les Salmonella. VI. Étude de l'antigène 1 présent sur deux Salmonella des groupes B et E. Ann. Inst. Pasteur, 1960, **98:**505–523.
81. Syverton, J. T., Ching, R. E., Cheever, F. S., and Smith, A. B.: Typhoid and paratyphoid A in immunized military personnel. J. Amer. Med. Assn., 1946, **131:**507–514.
82. Taylor, J.: Why christen a Salmonella? Internat. Bull. Bact. Nomen. Taxon., 1959, **9:**159–164.
83. Taylor, J., Lee, M. M., Edwards, P. R., and Ramsey, C. H.: A new type of flagellar variation associated with new antigens in the Salmonella group. J. Gen. Microbiol., 1960, **23:**583–588.
84. Thomson, S.: The number of bacilli harboured by enteric carriers. J. Hyg., 1954, **52:**67–70.
85. Tully, J. G., and Gaines, S.: H antigen of *Salmonella typhosa*. J. Bact., 1961, **81:**924–932.
86. Watson, K. C.: Isolation of *Salmonella typhi* from the blood stream. J. Lab. Clin. Med., 1955, **46:**128–134.
87. Watson, K. C.: Clot culture in typhoid fever. J. Clin. Path., 1954, **7:**305–307.
88. Webster, M. E., et al.: Studies on Vi antigen. IV. Physico-chemical characterization of Vi antigens isolated from V form Enterobacteraceae. J. Immunol., 1954, **73:**16–22.
89. Webster, M. E., Sagin, J. F., Landy, M., and Johnson, A. G.: Studies on the O antigen of *Salmonella typhosa*. I. Purification of the antigen. J. Immunol., 1955, **74:**455–465.
90. Weinstein, L., and Wasserman, E.: *Bacterium alcaligenes* (*Alcaligenes faecalis*) infections in man. New England J. Med., 1951, **244:**662–665.
91. Whiteside, R. E., and Baker, E. E.: The Vi antigens of the Enterobacteraceae. II. Immunologic and biologic properties. J. Immunol., 1959, **83:**687–696.

Chapter 22

THE ENTERIC BACILLI

The Dysentery Bacilli

Dysentery is a clinical rather than an etiological entity, and its characteristic symptoms, diarrhea, abdominal pain, and blood in the stools, may occur either alone or as part of the syndrome of a number of diseases. In the former instance dysentery may be of protozoan or bacterial etiology. In addition there is evidence suggesting that a filterable agent, presumably a virus, can cause dysentery in man. A dysentery-like infection may be produced by some members of the Salmonella group, but usually another group of bacteria, the dysentery bacilli, is responsible.[2]

The dysentery bacilli are gram-negative, non-spore-forming rods related to the other enteric bacteria. Some of them resemble the anaerogenic type of coliform bacilli and the typhoid bacillus in that they ferment carbohydrates with the production of acid and no gas. Others are related to the other coliform bacilli and paracolon bacilli. None of the dysentery bacilli are motile, and hence they do not contain the two types of antigens found in the paratyphoid bacilli. As a group they differ from one another biochemically and immunologically. In general, their uncertain relationship to the other enteric bacilli, coupled with their own heterogeneity, has made their classification a difficult matter.

The dysentery bacilli are facultative anaerobes, and their optimum temperature for growth is 37° C. Their nutritive requirements are not complex in that they will grow upon the ordinary nutrient (beef extract) mediums. In synthetic solutions nicotinic acid is apparently required by some strains, but whether amino acids are necessary is not known. They ferment glucose to much the same end products as the other enteric forms—lactic acid together with smaller amounts of formic and acetic acids and ethyl alcohol. Like the other gram-negative bacilli, they are relatively resistant to the bacteriostatic action of dyes, and these substances may be incorporated in differential mediums for their isolation; eosin–methylene blue agar is commonly used.

Classification. The dysentery bacilli are divided into two groups on the basis of the fermentation of mannitol, the non-mannitol fermenters including the Shiga bacillus. This distinction was of early importance and continues to be so in tropical regions and elsewhere where the Shiga bacillus occurs, for the dysentery produced by it is much more severe and has a higher case fatality rate than the other bacillary dysenteries, but is not of practical significance in this country where there is little or no Shiga dysentery. In addition to the Shiga bacillus, this group also includes the so-called parashiga bacilli and the Schmitz bacillus. The group of mannitol fermenters

is further subdivided on the basis of slow (4 to 7 days) fermentation of lactose and the fermentation of dulcitol and sorbitol.

In the past the nomenclature of the dysentery bacilli has been somewhat casual, and informal names such as the Shiga bacillus, the Flexner bacillus, the Strong bacillus, and the Hiss-Russell Y bacillus have had wide currency. The nomenclature has been further complicated, especially within the Flexner group, by various individual designations and by the vacillations of various formal systems of classification. The name Shigella for the genus has gained reasonably wide acceptance, and the dysentery bacilli are regarded as species of this single genus. Finally international agreement has been reached, based on species names and serotypes as suggested by Ewing,[23] to introduce a welcome order and stability.

Four groups of dysentery bacilli, designated A, B, C and D, each comprising a single species name with serotypes where appropriate, make up the genus Shigella. These are:

(1) The non-mannitol-fermenting bacilli:
Group A. *Shigella dysenteriae.* Including the Shiga dysentery bacillus, the Schmitz bacillus, and the Large-Sachs group of parashiga bacilli as immunologically independent serotypes.
(2) The mannitol-fermenting bacilli:
(a) Group B. *Shigella flexneri.* The Flexner dysentery bacilli, having characteristic cultural reactions and antigens in common, *i.e.*, group antigens, but differentiable into numbered serotypes.
(b) Group C. *Shigella boydii.* Culturally similar to the Flexner bacilli, but serologically unrelated to them or to one another, differentiable as independent numbered serotypes and including the Newcastle-Manchester bacilli.
(c) Group D. *Shigella sonnei.* The slow-lactose-fermenting dysentery bacilli, or Sonne-Duval bacilli, and including the bacilli formerly known as *Sh. ceylonensis A.*

These groups do not include various other bacilli found in association with diarrheal disease and described as dysentery bacilli, such as *Sh. alkalescens* and *Sh. dispar* which are considered to be coliforms, or bacilli such as *Sh. arabinotarda* types A and B which are identical with group A serotypes.

The nomenclature used by Russian workers differs from the foregoing,[64] a matter of some practical importance, but Ewing and his colleagues[22] have brought the Russian nomenclature into juxtaposition with the International Schema.

SHIGELLA DYSENTERIAE (Group A)

The dysentery bacilli making up this group are set apart by their inability to ferment mannitol. *Sh. dysenteriae* is immunologically heterogeneous, made up of 10 sharply separable serotypes arbitrarily designated by numbers. The serotypes appear to be unrelated antigenically except for unilateral cross reactions between some strains of types 2 and 6, and some strains of types 3 and 5. Two of the serotypes, 2 and 10, are serologically related to *Sh. boydii* type 1 (see below) through the sharing of two or more factors, and type 8 is divisible into subtypes, 8a and 8b.[21]

Shigella Dysenteriae Type 1. (*Bacterium dysenteriae, Shigella dysenteriae, Shigella shigae*). This was the first of the dysentery bacilli to be described. It was found by the Japanese bacteriologist Shiga to be the etiological agent of epidemic dysentery in Japan in 1898, and was found by Kruse in Germany two years later. It subsequently became known that this bacillus had been isolated ten years earlier by Chantemesse and Widal who found it in postmortem cultures of bowel contents and mesenteric lymph nodes, but it had become established as the Shiga bacillus or the Shiga-Kruse bacillus.

The Shiga bacillus differs from the other dysentery bacilli in its marked toxicity for man and experimental animals. Two kinds of toxin are formed: an endotoxin closely bound to the cell substance which is a polysaccharide-

Shigella Group A

Shigella dysenteriae

INTERNATIONAL TYPE	OLDER NOMENCLATURE		RUSSIAN NOMENCLATURE* NAME	SEROTYPE
1	*Sh. shigae*, Shiga-Kruse bacillus		Grigorjeff-Shiga	
2	Schmitz bacillus, *Sh. ambigua*		Stutzer-Schmitz	
3	Q 771		*Bact. dysenteriae* (Novgorodskaja-Semenova)	Roman
4	Q 1167	Large-Sachs group	*Bact. dysenteriae* (Novgorodskaja-Semenova)	1618
5	Q 1030		*Bact. dysenteriae* (Novgorodskaja-Semenova)	819
6	Q 454		*Bact. dysenteriae* (Novgorodskaja-Semenova)	Tjacht
7	Q 902		*Bact. dysenteriae* (Novgorodskaja-Semenova)	2435
8				
9	58			
10	2050–52			

* According to Ewing and Trabulsi.[25]

lipid-polypeptide complex[59, 60, 61] that appears to have considerable immunizing activity as assayed by mouse protection, and an exotoxin found in filtrates of broth cultures, protein in nature and thermolabile. The exotoxin (neurotoxin) has an effect upon the nervous system and produces paralysis,[8, 10, 51] while the endotoxin appears to act chiefly upon the alimentary tract. The neurotoxin is a true exotoxin[17] and resembles diphtheria toxin in a number of respects. It is produced most abundantly in aerated culture, and its formation is affected by the iron concentration of the medium; toxin formation is prevented by 0.01 M iron, and appreciably inhibited by as little as 0.000001 M. It has been prepared in highly purified form by van Heyningen and Gladstone[43, 44] and found to be similar to diphtheria toxin in potency and in possible mode of action. The relationship of this toxin to the pathogenesis of bacillary dysentery of *Sh. shigae* etiology is not at all clear. The exotoxin, but not the endotoxin, may be inactivated by formaldehyde and the toxoid used as an immunizing agent. It has not yet been possible to destroy the toxicity of this or other dysentery bacillus endotoxins and at the same time retain antigenicity.

Shiga bacillus infections have been observed most frequently in India, Japan, China, and other parts of Asia; they appear to be relatively rare in the United States.

Shigella Dysenteriae Type 2. (Schmitz bacillus, *Bacterium schmitzii, Shigella schmitzi, Shigella ambigua*). This serotype was described by Schmitz in 1917 as a cause of dysentery in a Rumanian war prison camp. It does not ferment mannitol but differs in that it produces indol and ferments sorbitol and rhamnose. The species is immunologically homogeneous except that freshly isolated strains contain two antigens, one of which is lost on continued cultivation, and antiserums prepared from stock strains may not agglutinate fresh strains too well. There is some cross-reaction, perhaps to one quarter titer, with the Shiga bacillus, but agglutinins are not reciprocally absorbed. The Schmitz bacillus is related serologically to *E. coli* O112. *Sh. dysenteriae* type 2 has been found in Europe, India, the Sudan, and elsewhere. In the United States it is not as common as some of the other dysentery bacilli but is encountered with some frequency and implicated in extensive institutional and other outbreaks of dysentery,[9, 53, 58, 68] and as an important cause of dysentery among chimpanzees in captivity.[36]

Other Serotypes. Strains of dysentery bacilli culturally identical with *Sh. dysenteriae* type 1 but immunologically unrelated were found by Dudgeon and

Urquhart in Macedonia in 1919 and designated by them *Bact. parashigae* (−) in contrast to the Schmitz bacillus which they termed *Bact. parashigae* (+). These bacilli have been observed from time to time in various parts of the world, including the United States, associated with diarrheal disease. They were studied in some detail by Large and by Sachs and are sometimes known as the Large-Sachs group or Sachs group of dysentery bacilli. Sachs distinguished eight immunological types, but three of these were paracolon bacilli, leaving five valid types in all. The Sachs types are Q771, Q1167, Q1030, Q454 and Q902; these are now designated *Sh. dysenteriae* types 3, 4, 5, 6 and 7.

Other non-mannitol-fermenting dysentery bacilli which differ immunologically from type 1 have been described. Of these the organism designated *Sh. arabinotarda* type A and Gober and Stacy strain 8524 has been found to be identical with Q771, and *Sh. arabinotarda* type B with Q1167. Still another, *Sh. wakefield,* is a paracolon bacillus. At the present time, then, *Sh. dysenteriae* is immunologically heterogeneous.

SHIGELLA FLEXNERI
(Group B)

Soon after Shiga's discovery, Flexner, working in the Philippines, discovered other dysentery bacilli which for a time were not clearly differentiated. Flexner's bacilli and those described by Strong and Musgrave in 1900 differed from *Sh. dysenteriae* both serologically and in the fermentation of mannitol. Attempts to subdivide the bacilli of the Flexner group by biochemical methods have not been successful; a wide variety may be separated on the basis of the variable fermentation of sucrose, dulcitol, sorbitol, maltose, raffinose, arabinose, inositol, and salicin, and indol formation, but such varieties are not correlated with immunological type and have had little practical value.

Shigella Flexneri (*Bacterium paradysenteriae,* Pseudodysentery bacillus, *Shigella paradysenteriae,* Flexner's bacillus, Hiss and Russell's Y bacillus, Strong's bacillus).

Sh. flexneri is made up of a group of immunological types that are distinct and yet related to one another. Five immunological types were distinguished by Andrewes and Inman on the basis of the distribution of four antigens, V, W, X and Z, which are designated as types V, W, X, Y, and Z. Subsequently Boyd reported evidence of the presence of type-specific and group-specific antigens in these types and in additional related forms and suggested that numbered types be substituted for the Andrewes and Inman types.

Further study[18] has fully substantiated this view, and the commonly accepted serological types of *Sh. flexneri* are

Figure 115. *Shigella flexneri.* Smear from a pure culture. Fuchsin; × 1050.

Shigella flexneri and Shigella boydii

INTERNATIONAL SCHEMA			OTHER NOMENCLATURE					
species name	sero-type	antigenic formula	Wheeler	Boyd	Andrewes-Inman	German	Russian	other
Sh. flexneri	1a	I: 3, 4, (7, 8)	I	V	V	B, C	$f(f_2)$	Flexner
	1b	I: (3, 4), 6	I		VZ	A	$f(f_1)$	
	2a	II: 3, 4	IIa	W	W	D	c	
	2b	II: 7, 8	IIb		WX	DX	b	
	3a	III: 6, 7, 8	III		Z	H	e	
	3b	III: (3, 4), 6, 7, 8						
	3c	III: (3, 4), 6					d	
	4a	IV: 3, 4	IV	103		F	$a(a_2)$; $a(a_1)$	
	4b	IV: (3, 4), 6	IV	103Z		J	$a(a_3)$	
	5	V: 7	V	P119		G	$g(g_1)$; $g(g_2)$	
	6	VI: (3, 4)	VI	88		L	Newcastle	*Sh. newcastle* Newcastle-Manchester bacilli
X variant		—: 7, 8	X					
Y variant		—: 3, 4	Y			Y1, Y2		Hiss-Russell
Sh. boydii	1			170			I	
	2			P288			V	
	3			D1				
	4			P274		R	III	
	5			P143			VII	
	6			D19				
	7					N, P	II	Lavington type T, *Sh. elousae* type 1296/7
	8							
	9						IV	
	10							
	11							
	12						VI	
	13							
	14							
	15							

shown in the accompanying table. The nomenclature of these types has become confused since different designations for the same type have been used by various authors in various countries. The common synonymous designations are shown in this table, and the types are indicated by Arabic numerals in accordance with the recommendation of the Shigella Commission to avoid confusion of Roman numerals with capital letters. As in the case of *Sh. dysenteriae,* the current Russian nomenclature differs from that of the International Schema; the correlations are shown in the accompanying table.

It may be noted here that certain non-mannitol-fermenting dysentery bacilli are closely related to the *Sh. flexneri* group on a serological basis and are, therefore, included in it. The Rio and Rabaul bacilli are *Sh. flexneri* type 4, but differ slightly in group antigens. The original Andrewes and Inman types are included in types 1, 2, and 3. Types 4 and 5 are the new types described by Boyd and originally called 103 and P119 respectively. Type 6, or Boyd 88, is not new but a variety of the Newcastle-Manchester bacillus that was first isolated from cases of dysentery in 1929. The X and Y types represent an as yet unsolved problem. These were so designated by Andrewes and Inman and apparently have no specific antigen; frequently cultures so labeled are found to be type 2,

and in other instances specific agglutination may be masked by rough antigens.

Serological typing is readily carried out, and phage typing, useful with staphylococci and Salmonellas in particular, has not been of general interest. It has been reported[38] that preliminary investigation of phage sensitivities has indicated a close correlation with serological groups.

Colonial morphology, as accentuated by oblique transmitted light, appears to be associated with both virulence and antigenic content.[15] Highly virulent colonial types contain a full complement of antigens, while morphologically distinct avirulent forms do not contain specific, or type, antigen, and are apparently transitional in the change from smooth to rough.

All of the Flexner bacilli are of worldwide distribution, the relative proportions varying from one locality to another. As a whole, *Sh. flexneri* is found in a large proportion of the dysentery cases in temperate climates, and even in tropical countries it is perhaps the most common of the dysentery bacilli. In this country, for example, 451 strains of a total of 769 isolated in routine examinations in Connecticut in 1940 to 1943 were *Sh. flexneri,* and of 1329 dysentery bacilli isolated from British and Indian troops in India, 999 were Flexner, 197 Shiga, 100 Schmitz, and 33 Sonne bacilli.

Sh. flexneri forms no soluble toxin or exotoxin but contains an endotoxin. It has been studied in some detail by Goebel and his co-workers,[6, 40] who have found that the somatic antigen which contains the toxicity is made up of a lipid component, a protein component, two carbohydrate components one of which contains labile acetyl groups, and a toxic component which Goebel believes to be a distinct substance and possibly associated with a purine or pyrimidine-like substance. In general, it has not been possible to detoxify the endotoxin without destroying its antigenicity. Mucinase is produced but to lower titer than by the cholera vibrio,[32] but whether this activity contributes to the pathogenicity of these microorganisms is not clear.

SHIGELLA BOYDII
(Group C)

Other mannitol-fermenting dysentery bacilli closely resembling *Sh. flexneri* in biochemical characteristics, but unrelated serologically either to the Flexner group or to one another, have been described. Six such immunological types described by Boyd as 170, P288, D1, D19, P143, and P274, and the bacillus described as *Sh. etousae* or Lavington I from the Mediterranean area during World War II, have been put into a single group as types of *Sh. boydii.* These are listed in the accompanying table as *Sh. boydii* types 1 to 7. Additional types have been described until there are now fifteen in all.[20] Some of these are related to coliform bacilli as well as to some of the other Boyd types; specifically types 10 and 11 are related to *E. coli* O105 and to *Sh. boydii* type 4.

The pathogenicity of these forms appears to be closely similar to that of *Sh. flexneri,* and their distribution seems to be ubiquitous. They have not, however, been studied in detail, as have, for example, the Flexner bacilli, with respect to chemical characterization of endotoxin and somatic antigens, effective immunity, etc.

Escherichia Alkalescens. *E. alkalescens* was described by Andrewes in 1918. Unlike the other dysentery bacilli, these bacilli ferment dulcitol. For some time they have been regarded as of uncertain pathogenicity, but evidence is accumulating which indicates that they are associated with disease. They have been found associated with sporadic enteric disease, and small epidemics are reported.[26] In the last few years *E. alkalescens* has been recognized more and more frequently in this country and appears to be more common than had been supposed. It is also pathogenic for experimental animals.

Strains of *E. alkalescens* have been regarded as biochemically distinct and immunologically homogeneous. Stuart, Rustigan, Zimmermann, and Corrigan,[73] however, have found that the species is made up of a graded series of biochem-

Figure 116. *Escherichia alkalescens.* Smear from a pure culture. Fuchsin; × 1050.

ical types. They have also demonstrated the presence of five antigens, two major antigens designated A and B, and three minor antigens, C, D, and E, in these bacilli. A, B, and C are present in all typical strains, while D and E occur singly or in combination to give four subtypes. They are immunologically related to the colon bacilli through the paracolon group and to Boyd P274. Two immunologically unrelated types have been described[63] and designated type II and type III in distinction to the original or type I. It has been suggested that type II be *Sh. tieté.* Both of these types include strains which ferment lactose.

There has been some tendency to put *E. alkalescens* together with *E. dispar* (see below) to make an alkalescens-dispar group of enteric bacilli set apart from the other Shigellas as more closely related to the coliform bacilli.[24]

SHIGELLA SONNEI
(Group D)

Lactose-fermenting dysentery bacilli discovered by Duval in 1904 have since been rediscovered by quite a number of observers. By force of usage these bacteria have come to be known as the Sonne type and are designated *Sh. sonnei* (Sonne-Duval bacillus, Duval's bacillus, Sonne's group III). The Sonne bacillus ferments mannitol and does not produce indol; it is serologically distinctive and homogeneous. There are two immunological types of *Sh. sonnei* which are designated I and II. Type I contains one antigen in predominance, while type II contains both antigens in equal amounts. The difference in agglutination titers is of practical importance, and type II antiserum is the one of choice. The two antigens differ in biochemical properties; that of type I is extracted from the bacilli in 50 per cent glycerol, while that of type II is not extracted in glycerol but in 7 M. urea. The rough form appears to be a variant of type II, but whether a succession of type I → type IIs → type IIr occurs is not altogether clear.[67] Type I tends to predominate in the acute disease and is mouse-virulent, while type II occurs for the most part in carriers and is not virulent for mice.[7]

The lactose fermentation is slow and may be delayed for a week or 10 days, and strains of this type were doubtless confused with Flexner bacilli by earlier workers. It is probably considerably more common in the United States than appears in the records. It has also been reported in warmer countries, and perhaps a more detailed study of the differentiation of the mannite-fermenting bacilli would increase the amount of dysentery properly ascribable to this microorganism.[74]

The slow lactose fermentation would appear to relate *Sh. sonnei* to members

of the colon group and in particular the so-called paracolon bacilli, but their immunological homogeneity tends to set them off.

Escherichia Dispar. Other lactose-fermenting dysentery bacilli were designated *Bact. dispar* by Andrewes. Some of Andrewes' strains were *Sh. sonnei,* but the type now termed dispar differs from the Sonne type in that sorbitol is fermented and indol is formed. *E. dispar* is serologically heterogeneous and not related to *Sh. sonnei* but appears to be related to certain Flexner strains. It has been found that a group of thirty-seven strains could be separated into three immunological types, two related to one another and the third independent. *E. dispar* may be divided into two varieties, *E. dispar* var. *ceylonensis* which ferments dulcitol, and *E. dispar* var. *madampensis* which does not.

BACILLARY DYSENTERY

Bacillary dysentery is a relatively common disease of man, more so in the warmer climates, and tends to be associated with crowding under conditions favoring spread of the infection from the human reservoir. It is more prevalent in backward countries, especially in the tropics and subtropics. It is a perennial problem in military operations, less so than in the pre-bacteriological days when, for example, more men died of diarrheal disease during the Civil War in this country than were killed in battle. As a seriously incapacitating disease tending to occur in epidemic form, it continued to be a problem in World War I when, for instance, it was a significant element in the outcome of the battle of Gallipoli, during World War II especially in the South Pacific and Mediterranean areas, and subsequently in situations such as the landing of American troops in Lebanon in the the late 1950's. While the proportion of Shiga bacillus infections has sharply declined to reduce mortality, bacillary dysentery remains an important incapacitating disease.

The infection remains a local one in the bowel with invasion of little more than the superficial tissues, and the debilitating effects are attributable largely to the loss of fluid and electrolyte consequent to the purging diarrhea. There is no doubt of the etiological relation of the dysentery bacilli to the disease; in addition to the presence of enormous numbers in the acute disease, the immune response as serum agglutinin, and the production of analogous disease in certain experimental animals (see below), accidental infections of man are well known and the disease has been produced in human volunteers.[70, 71]

Pathogenicity for Man.[77] The incubation period of bacillary dysentery is generally short, about 48 hours, and the disease may be acute or tend to run a chronic course. Apart from the inflammatory, sometimes ulcerative or diphtheritic, lesions in the intestine (ulcerative colitis), the anatomical picture of dysentery presents little that is characteristic. The liver abscesses that are found, as a rule, in amebic dysentery are absent in the bacterial diseases, one series having been reported of 1130 cases of bacillary dysentery without a single abscess. Dysentery bacilli are sometimes found in immense numbers in the dejecta, often in almost pure culture. They may be found at autopsy in the mesenteric glands, but, as a rule, not in the spleen or other internal organs, nor do they commonly occur in the blood or urine. Bacillary dysentery is, therefore, not a septicemia but an infection localized in the alimentary tract, in this respect resembling Asiatic cholera rather than typhoid fever. Recurrent diarrheal disease, often called chronic ulcerative colitis, may be caused by dysentery bacilli, indicating that the bacilli may persist in the bowel, possibly in the superficial layers of the intestinal epithelium, for long periods.[16, 27]

The dysentery toxin is excreted in rabbits, and probably in man, by the large intestine. The selective action of the

toxin upon the tissues, rather than any local action of the bacilli themselves, appears to be responsible for the inflammation and other local changes. When the toxin is introduced directly into the gut, no symptoms are produced, suggesting that the toxin primarily affects the deeper cells rather than the surface of the mucous membrane.[27]

In the large series of cases studied in Denmark caused by the Sonne and Flexner types the case fatality was about 2 per cent. The Shiga bacillus dysentery of the tropics is much more often fatal (20 per cent). The clinical disease is considerably more severe in the Shiga infections than in Flexner infections, and complications, such as arthritis, are almost invariably associated with the former type of infection.

Some portion, apparently an increasing portion, of infant diarrhea is of dysentery bacillus etiology. Certain kinds of bacillary dysentery, especially *Sh. sonnei* infections, have become more common, and the epidemiology of the disease has changed so that the disease seems to be shifting from "the scourge of armies to the bane of nurseries,"[74] where it may assume a fulminating, rapidly fatal form.[49] Some investigators have isolated the dysentery bacilli from the excreta, particularly in those cases in which there is mucus in the stools. Those cases with which dysentery bacilli are associated do not appear to differ clinically from those in which they are not found, and it is uncertain just what proportion of cases of infant diarrhea is caused by the dysentery bacilli.

Carriers. It is probable that dysentery bacillus infection is very common, many of the infections going unrecognized because of the mildness of the symptoms. Persons with such infections are, of course, convalescent carriers who continue to discharge the bacilli for an average time of 3 to 5 weeks. It is not clear whether a permanent chronic carrier state analogous to the chronic typhoid bacillus carrier occurs, but many convalescents continue to discharge dysentery bacilli over long periods, and inapparent infection is not uncommon. In any case, it is the casual carrier and changing groups of casual carriers or ambulatory cases that are of primary importance in the maintenance and spread of the infection, and the disease persists in smoldering, endemic form.[45, 46]

Bacteriological Diagnosis.[50] Since, as indicated earlier, dysentery is a clinical rather than an etiological entity, the causative microorganism must be isolated and identified to allow a diagnosis of bacillary dysentery. The bacilli may be found in fecal specimens, and rectal swabs are cultured for the detection of

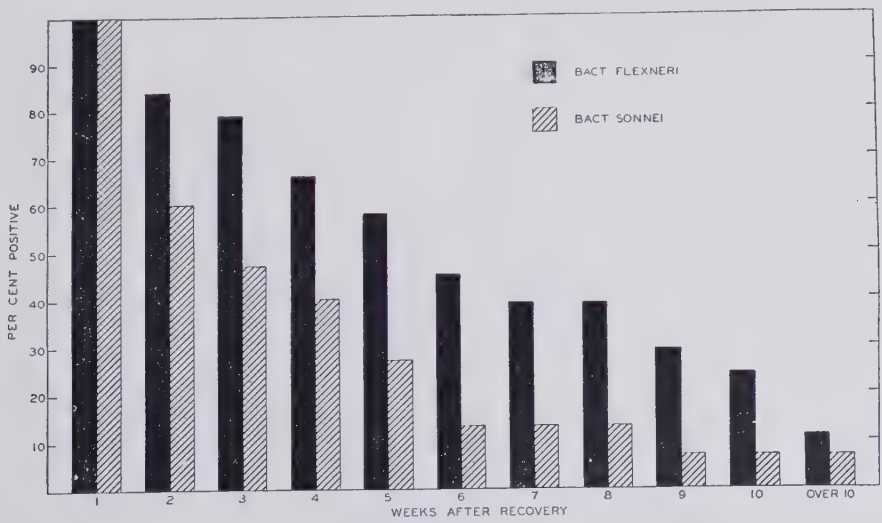


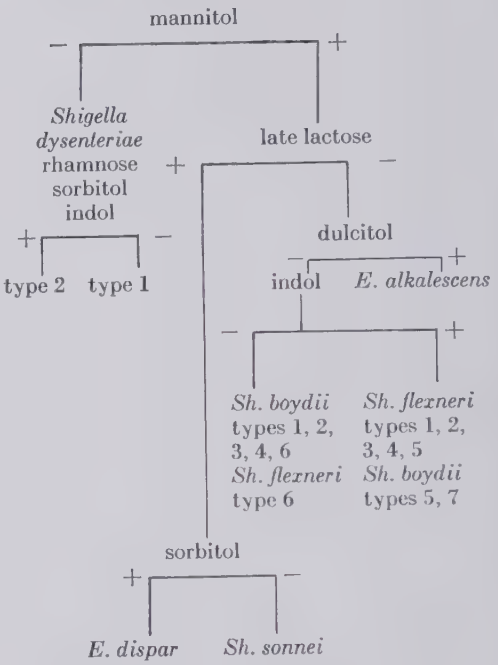
Figure 117. The persistence of dysentery bacilli in the feces of convalescents. Prepared from data of Watt, Hardy, and DeCapito.[79]

carriers. The methods of enrichment culture and direct plating are essentially those used in the isolation of Salmonella and the typhoid bacillus. Desoxycholate-citrate and S-S agars are the most useful of the agar mediums; bismuth-sulfite is not suitable because species other than *Sh. flexneri* are inhibited. Colonies are subcultured and identified by biochemical reactions and slide agglutination. For tentative serological identification polyvalent serums may be used, including (1) polyvalent Shiga serum, (2) polyvalent Flexner serum, (3) polyvalent *Sh. boydii* serum, (4) *Sh. sonnei* types I and II, (5) *E. dispar* types, and (6) *E. alkalescens*. The cross-reaction between these groups of antiserums may be removed by agglutinin absorption.[19] For precise identification monospecific antiserums are, of course, required.

Chemotherapy.[37, 48, 76] The sulfonamides have been found to be effective chemotherapeutic agents in bacillary dysentery; the soluble compounds, such as sulfadiazine, are more effective than the nonabsorbable compounds, probably because the bacilli penetrate into the intestinal wall and regional lymph nodes. Sulfonamide-fast strains of dysentery bacilli are encountered with apparently increasing frequency and antibiotic therapy is of considerable importance. Of these, streptomycin, chloramphenicol, and the tetracyclines have been found to be effective, but the dysentery bacilli, like most other gram-negative bacteria, are relatively insusceptible to penicillin.

Epidemiology. Infections caused by dysentery bacilli are probably far more common than is generally recognized. In 1945, 33,495 cases and 400 deaths were reported from thirty-eight states, rates of 32.4 and 0.4 per 100,000 respectively. Attacks of severe illness grade off into mild and almost trivial attacks of simple diarrhea. Several outbreaks of typical "food poisoning" attributed to the Sonne bacillus are on record. In a number of localities where careful bacteriological studies have been made, dysentery bacilli have been found widely distributed both in patients with gastrointestinal derangements and in the general

Biochemical Separation of the Dysentery Bacilli



population. Probably the most important single reservoir of infection is the human carrier, either convalescent or with inapparent infection. The extent to which the carrier state occurs has been appreciated only in recent years. In a study of dysentery bacillus infection in the normal population Watt and Hardy[78] found *Sh. flexneri* in 11 per cent of the population in New Mexico, 4 per cent in Puerto Rico, 3 per cent in Georgia, and 0.1 per cent in New York City, with an estimated annual morbidity of 60 per cent in Puerto Rico, 48 per cent in New Mexico, and 20 per cent in Georgia, and an over-all ratio of convalescent or passive carriers to cases of 9:1.

Dysenteric infections seem to be most common in hot countries and in the summer months in temperate climates, although they may occur at any season of the year. The acute diarrheal disease occurring routinely in tropical countries, and known locally as "Gyppy tummy," "Delhi belly," etc., are frequently bacillary dysentery, the Flexner and Boyd types appearing to be the most common where studies have been made.[28, 29] The spread of the disease is due to the more

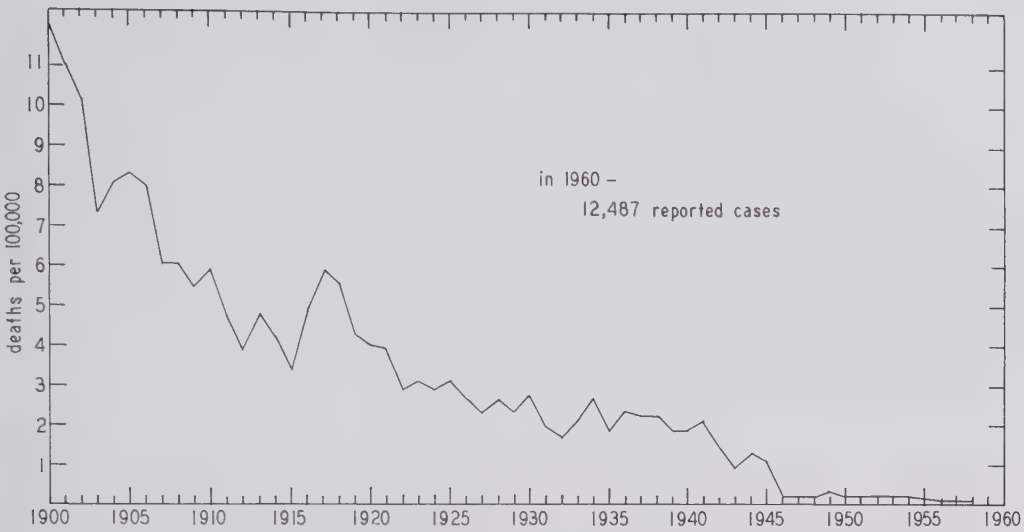


Figure 118. The prevalence of bacillary dysentery as shown by the mortality rates in the Registration Area. Data from the Morbidity and Mortality Weekly Report, vol. 9, 1961. Communicable Disease Center, Public Health Service, U. S. Department of Health, Education and Welfare.

or less direct transfer of the specific bacillus from infected intestinal discharges to the alimentary tract of a fresh individual. Polluted water may play a part in some outbreaks but is apparently not nearly so important a factor in dysentery as it is in typhoid fever. Improper disposal of excreta permitting dissemination by flies, and the contamination of food by chronic carriers and convalescents, appear to be the most important factors in the spread of bacillary dysentery. The role of insects, especially flies, has been demonstrated,[45] and is probably an important one, and the seasonal incidence of bacillary dysentery is in keeping with this. In the epidemic reported by Kuhns and Anderson infected flies were caught in kitchens and operating latrines; similar reports are not uncommon. The decline in diarrhea and enteritis and the shifting proportions of the etiologic agents of the last few decades is very likely a reflection of the general improvement in sanitary conditions.[75]

At the present time in temperate climates dysentery flourishes especially in mental hospitals and other large institutions, where lack of personal hygiene among the inmates favors the transfer of infection. Whenever it gains a foothold in these institutions it seems to be kept alive chiefly by chronic carriers and proves an obstinate problem. Weekly bacteriological examination in one institution showed that more than 50 per cent of the dysentery patients continued to excrete dysentery bacilli for long periods—in one case over 4½ years. Epidemic bacillary dysentery is also a disease of armies in the field, where opportunities for the dissemination of infection are frequently very great, and extensive outbreaks are common.

Although in small outbreaks a single type of dysentery bacillus may be found, in the larger outbreaks more than one type is almost always observed. The most common types in this country appear to be Flexner, Sonne, Schmitz and *E. alkalescens* in that order.

Immunity. A significant element in resistance to infection with dysentery bacilli appears to be the protective effect of the normal bowel flora, *i.e.*, the infecting microorganism must compete successfully to establish a focus of infection with multiplication and coincident toxin formation. While the infection is probably established in the small bowel, antagonisms between bacteria occurring as a part of the intestinal flora are demonstrable *in vitro* under experimental conditions which simulate the *in vivo* environment.[35, 42, 66] It has been

possible, too, to produce experimental enteric infections in the guinea pig by the use of streptomycin-resistant dysentery bacilli given by the intragastric route in an inoculum containing the antibiotic.[34]

The development of an immunity effective to some degree is indicated by the relative resistance of the resident population of an endemic area to the acute disease which affects recent arrivals, *e.g.* "acclimatization diarrhea."[29] This phenomenon is well known to residents of temperate climates visiting tropical and subtropical areas.

Antibodies, agglutinins, are formed in response to infection with dysentery bacilli, usually appearing after the sixth day. The titer is relatively low as a rule. The diagnostic significance of agglutinins is somewhat uncertain largely because "normal" agglutinins are common. Normal serum commonly agglutinates *Sh. dysenteriae* 1 in 1:20 dilution, but a titer of 1:40 or higher is suggestive of infection. Agglutinins for *Sh. flexneri* occur in much higher titer, as high as 1:150 in normal serum, and the titer is frequently increased in infections with other species of dysentery bacilli. Flexner agglutinin has, then, very little diagnostic value unless it is to high titer, and agglutinins for Shiga and typhoid bacilli are absent. Agglutinins to the Newcastle bacillus, *Sh. dysenteriae* 2, and *E. alkalescens* occur only in low titer. *Sh. sonnei* is often agglutinated to titers as high as 1:50 by normal serum, and, though the titer may occasionally be very high in infection; sometimes little or no agglutinin response is apparent.

The antibody demonstrable as agglutinin, and as protective antibody by the mouse protection test, is apparently unrelated to effective immunity.[13, 14] Consistent with this, vaccine prophylaxis of bacillary dysentery has been almost uniformly ineffective.[41, 47, 70] In this connection it may be recalled that the infection is confined to the lumen of the intestine, intestinal mucosa, and lymphoid tissue as a rule, and in a very real sense remains outside the body. Coproantibody has been demonstrated in bacillary dysentery,[1] but its part, if any, in effective immunity is uncertain. Bacillary dysentery, then, differs from typhoid fever in the important respect that the bacilli do not invade the tissues and consequently are not exposed to the action of circulating antibody and phagocytic cells. Possibly this accounts, at least in part, for consistent failure to develop an effective immunity to infection with these bacilli.

Pathogenicity for Lower Animals. Although occasional isolations have been reported from lower animals, such as the dog, the dysentery bacilli occur primarily in man. Monkeys and chimpanzees in captivity are an exception for they are commonly infected, usually with *Sh. flexneri* but also with other species. The infection is latent for the most part but may assume an acute form. It is probably acquired from man and spread from animal to animal in crowded cages; when special precautions are taken, from the time of capture, to avoid contact and prevent dissemination, these animals are often free of the infection.

A fatal infection may be produced in the conventional laboratory animals by intraperitoneal inoculation; the mouse is one of the most susceptible. Such infections are systemic and hardly more than distantly related to the naturally occurring human disease and have limited applicability although they are useful in titrating protective antibody, etc.[13]

Other infections more nearly approximating the human disease may be produced. The first of these was that devised by Bingel[3, 4, 5] by instillation of dysentery bacilli, or other enteric bacilli, into the urinary bladder of female guinea pigs. The resulting infection simulates some aspects of the naturally occurring enteric infection, but does not remain completely localized. Another kind of localized infection is the keratoconjunctivitis that may be produced in the guinea pig by instillation of suspensions of the bacilli; it has been studied by Russian workers[39, 55, 69] and also in Europe[66] and in this country.[54] This infection also simulates to a degree the naturally occurring disease.

In general attempts to produce an

enteric infection in experimental animals by oral or intragastric inoculation have not been successful, but within the last few years a number of such infections have been produced. This kind of infection has been produced in mice,[11, 12, 56, 57] cats have been so infected by Russian workers,[52, 62, 72] and fatal infections have been produced in the guinea pig.[30, 31, 33, 34] These are infections with *Sh. flexneri* for the most part and, while some strains appear highly virulent, most commonly enteric infection is produced only by the introduction of additional factors. These include the use of streptomycin-resistant bacteria in an inoculum containing the antibiotic, starvation, preliminary administration of alkali, the administration of laudanum or morphine to inhibit intestinal motility, partial poisoning with Shiga toxin, carbon tetrachloride, etc. In spite of these complications such infections have been extremely useful as models approximating the human disease.

REFERENCES

1. Barksdale, W. L., and Ghoda, A.: Agglutinating antibodies in serum and feces. J. Immunol., 1951, **66:**395–401.
2. Bensted, H. J.: Dysentery bacilli—Shigella. A brief historical review. Canadian J. Microbiol., 1956, **2:**163–174.
3. Bingel, K. F.: Eine tierexperimentelle Methode zum Studium der Infektion mit gramngeativen Darmbakterien, insbesondere der Ruhr. Ztschr. f. Hyg. u. Infektionskr., 1943, **125:**110–122.
4. Bingel, K. F.: Prophylaxe der Ruhr im Tierexperiment. Ztschr. f. Hyg. u. Infektionskr., 1944, **125:**574–609.
5. Bingel, K. F.: Tierexperimentelle Beiträge zur Pathogenese der Ruhr. Ztschr. f. Hyg. u. Infektionskr., 1944, **125:**610–650.
6. Binkley, F., Goebel, W. F., and Perlman, E.: Studies on the Flexner group of dysentery bacilli. II. Chemical degradation of the specific antigen of type Z *Shigella paradysenteriae* (Flexner). J. Exptl. Med., 1945, **81:**331–347.
7. Branham, S. E., Carlin, S. A., and Riggs, D. B.: A comparison of the incidence of phases I and II of *Shigella sonnei* in cultures from acute infections and carriers. Amer. J. Pub. Health, 1952, **42:**1409–1413.
8. Bridgewater, F. A. J., Morgan, R. S., Rowson, K. E. K., and Wright, G. P.: The neurotoxin of *Shigella shigae*. Morphological and functional lesions produced in the central nervous system of rabbits. Brit. J. Exptl. Path., 1955, **36:**447–453.
9. Burkhardt, F., and Legler, F.: Bakteriologische und serologische Beobachtungen bei einer Anstaltsendemie durch Schmitz-Ruhrbakterien (*Shigella dysenteriae* 2). Arch. f. Hyg. u. Bakt., 1958, **142:**186–193.
10. Cavanagh, J. B., Howard, J. G., and Whitby, J. L.: The neurotoxin of *Shigella shigae*. A comparative study of the effects produced in various laboratory animals. Brit. J. Exptl. Path., 1956, **37:**272–278.
11. Cooper, G. N.: Experimental shigellosis in mice. I. Chronic infection with *Shigella dysenteriae* type 2. Australian J. Exptl. Biol. Med. Sci., 1959, **37:**193–200.
12. Cooper, G. N., and Pillow, J. A.: Experimental shigellosis in mice. II. Immunological responses to *Shigella dysenteriae* type 2 infection. Australian J. Exptl. Biol. Med. Sci., 1959, **37:**201–209.
13. Cooper, M. L., and Keller, H. M.: Studies in dysentery vaccination. I. The passive mouse protection test. J. Immunol., 1948, **58:**349–356.
14. Cooper, M. L., and Keller, H. M.: Studies in dysentery vaccination. II. Humoral antibody content of sera from children convalescent from dysentery. J. Immunol., 1948, **58:**357–360.
15. Cooper, M. L., Keller, H. M., and Walters, E. W.: Microscopic characteristics of colonies of *Shigella flexneri* 2a and 2b and their relation to antigenic composition, mouse virulence and immunogenicity. J. Immunol., 1957, **78:**160–171.
16. El Ghaffar, Y. A., and El Ghaffar, M. A.: Atypical chronic intestinal shigellosis. Amer. J. Trop. Med. Hyg., 1955, **4:**301–309.
17. Engley, F. B., Jr.: The neurotoxin of *Shigella dysenteriae* (Shiga). Bact. Rev., 1952, **16:**153–178.
18. Ewing, W. H.: Shigella nomenclature. J. Bact., 1949, **57:**633–638.
19. Ewing, W. H.: Shigella grouping antiserums. J. Lab. Clin. Med., 1950, **36:**471–472.
20. Ewing, W. H., and Hucks, M. S.: Four new provisional serotypes of Shigella. J. Immunol., 1952, **69:**575–580.
21. Ewing, W. H., and Johnson, J. G.: Intersubgroup and intrasubgroup relationships within the genus Shigella: Subgroup A. Canadian J. Microbiol., 1961, **7:**303–308.
22. Ewing, W. H., Johnson, J. G., and Davis, B. R.: A comparison of the Shigella classification used in the U.S.S.R. with the International Shigella Schema. Internat. Bull. Bact. Nomen. Taxon., 1959, **9:**177–181.
23. Ewing, W. H., Reavis, R. W., and Davis, B. R.: Provisional shigella serotypes. Canadian J. Microbiol., 1958, **4:**89–107.
24. Ewing, W. H., Taylor, M. C., and Hucks, M. W.: The alkalescens-dispar group. Pub. Health Repts., 1950, **65:**1474–1480.
25. Ewing, W. H., and Trabulsi, L. R.: Further studies with cultures of Shigella classified according to the method used in the U.S.S.R. Internat. Bull. Bact. Nomen. Taxon, 1962, **12:**1–8.
26. Felsen, J., and Wolarsky, W.: Bacillary dysentery due to *shigella alkalescens*. Arch. Intern. Med., 1952, **89:**428–430.
27. Felsen, J., and Wolarsky, W.: Acute and chronic bacillary dysentery and chronic ulcerative colitis. J. Amer. Med. Assn., 1953, **153:**1069–1072.

28. Floyd, T. M.: *Shigella boydii* serotypes in Egypt. J. Bact., 1956, **71:**525–529.
29. Floyd, T. M.: A study of the etiology of "acclimatization diarrhea" among Americans in Egypt. Amer. J. Trop. Med. Hyg., 1956, **5:**516–520.
30. Formal, S. B., Dammin, G. J., Labrec, E. H., and Schneider, H.: Experimental Shigella infections: Characteristics of a fatal infection produced in guinea pigs. J. Bact., 1958, **75:** 604–610.
31. Formal, S. B., Dammin, G. J., Schneider, H., and Labrec, E. H.: Experimental Shigella infections. II. Characteristics of a fatal infection in guinea pigs following the subcutaneous inoculation of carbon tetrachloride. J. Bact., 1959, **78:**800–804.
32. Formal, S. B., and Lowenthal, J. P.: Distribution of mucinolytic activity in strains of Shigella. Proc. Soc. Exptl. Biol. Med., 1956, **92:** 10–12.
33. Formal, S. B., Noyes, H. E., and Schneider, H.: Experimental Shigella infections. III. Sensitivity of normal, starved and carbon tetrachloride treated guinea pigs to endotoxin. Proc. Soc. Exptl. Biol. Med., 1960, **103:**415–418.
34. Freter, R.: Experimental enteric Shigella and Vibrio infections in mice and guinea pigs. J. Exptl. Med., 1956, **104:**411–418.
35. Freter, R.: In vivo and in vitro antagonism of intestinal bacteria against *Shigella flexneri*. II. The inhibitory mechanism. J. Infect. Dis., 1962, **110:**38–46.
36. Galton, M. M., Mitchell, R. B., Clark, G., and Riesen, A.: Enteric infections in chimpanzees and spider monkeys with special reference to a sulfadiazine resistant Shigella. J. Infect. Dis., 1948, **83:**147–154.
37. Garfinkel, B. T. et al.: Antibiotics in acute bacillary dysentery. Observations in 1,408 cases with positive cultures. J. Amer. Med. Assn., 1953, **151:**1157–1159.
38. Gaspar, G.: Correlations between the antigenic structure and phage sensitivity of types of group *Shigella flexneri*. Acta Microbiol., 1958, **5:**243–251.
39. Gekker, V. D., Belaya, J. A., and Melnik, E. T.: Experimental keratoconjunctivitis as a model for the study of dysentery. J. Hyg. Epidemiol. Microbiol. Immunol., 1957, **1:**70–74.
40. Goebel, W. F., Binkley, F., and Perlman, E.: Studies on the Flexner group of dysentery bacilli. I. The specific antigens of *Shigella paradysenteriae*. J. Exptl. Med., 1945, **81:**315–330.
41. Hardy, A. V., and Halbert, S. P.: Studies of the acute diarrheal diseases. XIX. Immunization in shigellosis. Pub. Health Repts., 1948, **63:** 685–688.
42. Hentges, D. J., and Freter, R.: In vivo and in vitro antagonism of intestinal bacteria against *Shigella flexneri*. I. Correlation between various tests. J. Infect. Dis., 1962, **110:**30–37.
43. van Heyningen, W. E., and Gladstone, G. P.: The neurotoxin of *Shigella shigae*. 3. The effect of iron on production of the toxin. Brit. J. Exptl. Path., 1953, **34:**221–229.
44. van Heyningen, W. E., and Gladstone, G. P.: The neurotoxin of *Shigella shigae*. 4. A semimicro method for the flocculation assay of the toxin. Brit. J. Exptl. Path., 1953, **34:**230–231.
45. Higgins, A. R., and Floyd, T. M.: Studies in shigellosis. I. General considerations, locale of studies and methods. Amer. J. Trop. Med. Hyg., 1955, **4:**263–270.
46. Higgins, A. R., Floyd, T. M., and Kader, M. A.: Studies in shigellosis. II. Observations on incidence and etiology of diarrheal disease in Egyptian village children. Amer. J. Trop. Med. Hyg., 1955, **4:**271–280.
47. Higgins, A. R., Floyd, T. M., and Kader, M. A.: Studies in shigellosis. III. A controlled evaluation of a monovalent Shigella vaccine in a highly endemic environment. Amer. J. Trop. Med. Hyg., 1955, **4:**281–288.
48. Higgins, A. R., Floyd, T. M., and Kader, M. A.: Studies in shigellosis. IV. A controlled trial of sulfadiazine, dihydrostreptomycin and oxytetracycline as long term prophylactic agents in a highly endemic environment for shigellosis. Amer. J. Trop. Med. Hyg., 1955, **4:**289–300.
49. Hoefnagel, D.: Fulminating, rapidly fatal shigellosis in children. New England J. Med., 1958, **258:**1256–1257.
50. Hormaeche, E., and Peluffo, C. A.: Laboratory diagnosis of Shigella and Salmonella infections. Bull. W. H. O., 1959, **21:**247–277.
51. Howard, J. G.: Observations on the intoxication produced in mice and rabbits by the neurotoxin of *Shigella shigae*. Brit. J. Exptl. Path., 1955, **36:**439–446.
52. Khomik, S. R.: The cat as an experimental model in the study of dysentery. J. Microbiol. Epidemiol. Immunobiol., 1957, **28:**526–529. (Translated from the Russian.)
53. King, W. E., and French, E. L.: An outbreak of dysentery caused by *Shigella schmitzi*. Med. J. Australia, 1947, **2:**136–138.
54. Mackel, D. C., Langley, L. F., and Venice, L. A.: The use of the guinea-pig conjunctivae as an experimental model for the study of virulence of Shigella organisms. Amer. J. Hyg., 1961, **73:**219–223.
55. Manolov, D. G.: A study of some questions of pathogenicity and immunity in dysentery using experimental models of Shigella keratoconjunctivitis in guinea pigs. J. Hyg. Epidemiol. Microbiol. Immunol., 1957, **1:**322–328.
56. McGuire, C. D., and Floyd, T. M.: Studies on experimental shigellosis. I. Shigella infections of normal mice. J. Exptl. Med., 1958, **108:**269–276.
57. McGuire, C. D., and Floyd, T. M.: Studies on experimental shigellosis. II. Effect of fasting and fatigue on *Shigella flexneri* 3 infections in mice. J. Exptl. Med., 1958, **108:**277–282.
58. Medical Research Council: An outbreak of dysentery due to Schmitz's bacillus. Monthly Bull. Pub. Health Lab. Service, 1943, **2:**52.
59. Morgan, W. T. J.: Studies in immunochemistry. II. The isolation and properties of a specific antigenic substance isolated from *B. dysenteriae* (Shiga). Biochem. J., 1937, **31:**2003–2021.
60. Morgan, W. T. J., and Partridge, S. M.: Studies in immunochemistry. 4. The fractionation and nature of antigenic material isolated from *Bact. dysenteriae* (Shiga). Biochem. J., 1940, **34:**169–191.
61. Morgan, W. T. J., and Partridge, S. M.: Studies in immunochemistry. 6. The use of phenol and alkali in the degradation of antigenic material isolated from *Bact. dysenteriae* (Shiga). Biochem. J., 1941, **35:**1140–1163.
62. Morgunov, I. N., Maksimovich, N. A., and Yagud, S. L.: The search for an experimental

model of dysentery infection. I. Reproduction of dysentery infection in cats by preliminary sensitization. J. Microbiol. Epidemiol. Immunobiol., 1959, **30:**24–31. (Translated from the Russian.)

63. Neter, E.: Antigenic types of *Shigella alkalescens*. Proc. Soc. Exptl. Biol. Med., 1944, **57:**200–205.
64. Novgorodskaia, E. M.: Considerations on the classification of the dysentery bacilli. J. Microbiol. Epidemiol. Immunobiol., 1959, **30:**7–17. (Translated from the Russian.)
65. Piechaud, M., Szturm-Rubinsten, S., and Piechaud, D.: Evolution histologique de la keratoconjunctivite à bacilles dysenterique du cobaye. Ann. Inst. Pasteur, 1958, **94:**298–309.
66. Ransom, J. P., Finkelstein, R. A., Ceder, R. E., and Formal, S. B.: Interactions of *Vibrio cholerae, Shigella flexneri,* enterococci and lactobacilli in continuously fed cultures. Proc. Soc. Exptl. Biol. Med., 1961, **107:**332–336.
67. Rauss, K., Ketyi, I., Vertenyi, A., and Voros, S.: Studies on the nature of phase variation of *Sh. sonnei*. Acta Microbiol. Acad. Sci. Hungaricae, 1961, **8:**53–63.
68. Schleifstein, J., and Coleman, M. B.: An extensive outbreak of enteric disease incited by *B. dysenteriae* Schmitz. J. Infect. Dis., 1937, **61:**257–258.
69. Sereny, B.: Experimental keratoconjunctivitis shigellosa. Acta Microbiol., 1957, **4:**367–376.
70. Shaughnessy, H. J., et al.: Experimental human bacillary dysentery. J. Amer. Med. Assn., 1946, **132:**362–368.
71. Shaughnessy, H. J., et al.: Bacillary dysentery in human volunteers. Yale J. Biol. Med., 1947, **19:**537–546.
72. Sirotinin, N. N., Ousierskaia, I. V., Brodskaia, E. A., and Gromashevskaia, L. L.: An experimental model of dysentery infection. J. Microbiol. Epidemiol. Immunobiol., 1958, **29:**346–351. (Translated from the Russian.)
73. Stuart, C. A., Rustigian, R., Zimmermann, A., and Corrigan, F. V.: Pathogenicity, antigenic relationships and evolutionary trends of *Shigella alkalescens*. J. Immunol., 1943, **47:**425–437.
74. Taylor, I.: The changing epidemiology of Sonne dysentery. Proc. Roy. Soc. Med., 1957, **50:** 31–36.
75. Taylor, J.: The diarrhoeal diseases in England and Wales with special reference to those caused by Salmonella, Escherichia and Shigella. Bull. W. H. O., 1960, **23:**763–779.
76. Taylor, P. J.: Acute bacillary dysentery in Cyprus. A comparative study of treatment in 332 cases. Brit. Med. J., 1959, **ii:**9–12.
77. Watkins, H. M. S.: Some attributes of virulence in Shigella. Ann. N. Y. Acad. Sci., 1960, **88:** 1167–1186.
78. Watt, J., and Hardy, A. V.: Studies of the acute diarrheal diseases. X. Cultural surveys of normal population groups. Pub. Health Repts., 1945, **60:**261–273.
79. Watt, J., Hardy, A. V., and De Capito, T.: Studies of the acute diarrheal diseases. VII. Carriers of *Shigella dysenteriae*. Pub. Health Repts., 1942, **57:**524–529.

Chapter 23

THE CHOLERA VIBRIO AND RELATED FORMS

Although Asiatic cholera has doubtless smoldered endemically in parts of India for many centuries, the year 1817 marked its first considerable extension beyond the borders of that country. Europe was first invaded in 1831, and since that date a series of great epidemics has carried the disease over a large part of the civilized world. The disease was brought to New York by Irish immigrants during the pandemic of 1832–33 and in the pandemic of 1846–62 invaded the United States via New Orleans (1848) and spread up the Mississippi Valley. The fourth great pandemic, that of 1864–75, affected Asia, Africa, Europe and America. The causal agent of the disease, the cholera vibrio, was discovered in 1883 by Koch in the intestinal discharges of cholera patients. Similar microorganisms were described by later workers in infected water and elsewhere, and now a number of species are known. In general, however, these other vibrios are nonpathogenic and have been studied in terms of their relation to the cholera vibrio and are, therefore, not particularly well known. Of what is now a fairly sizable group, only a few species are pathogenic: the microorganism discovered by Koch and variously termed *Spirillum cholerae asiaticae* (Koch), *S. cholerae, Vibrio cholerae,* the comma bacillus, and *V. comma* (Bergey); certain of the El Tor vibrios; *Vibrio fetus* infecting domestic animals; and a vibrio pathogenic for pigeons and guinea pigs, *Vibrio metchnikovii.*

VIBRIO CHOLERAE[87]

Morphology and Staining. The cholera vibrio is a short, slightly curved and twisted rod, 1.5 to 3 μ in length and 0.4 to 0.6 μ in breadth. It may occur singly or in chains which have the appearance of short spirals or S-shaped forms (two cells). The straight and spiral threads formed in the pellicle of liquid gelatin cultures are usually regarded as involution forms. Cultures that have been maintained for a long time on agar often lose the curved form and appear as straight rods but resume the more characteristic form when passed through animals. The vibrios are actively motile by a single polar flagellum which is shorter than the flagella of most bacteria. Spores are not formed. The cholera vibrio stains readily with the ordinary aniline dyes and is gram-negative.

Colonies on agar mediums are similar to those of the other enteric bacilli but may be distinguished from those of *Escherichia coli* by their thin, opalescent appearance. They are 1 to 2 mm. in diameter, low, convex and grayish yellow in color, with a finely granular consist-

ency which is accentuated under low magnification. Some strains are hemolytic on blood agar while others are not (see below).

Physiology. The cholera vibrio is strongly aerobic, and only very sparse growth appears under anaerobic conditions, and then on prolonged incubation. It grows over the temperature range of 16° to 42° C. with an optimum growth at 37° C. An alkaline reaction is essential for good growth; the bacteria will grow over a pH range of 6.4 to 9.6 and are usually cultivated at an alkaline reaction, pH 7.8 to 8.0. This marked tolerance for alkali is taken advantage of in the preparation of selective mediums for the isolation of the cholera vibrio, the pH of such mediums usually being about 9.5. They are not nutritionally fastidious and may be grown in peptone water. Many strains grow on simple synthetic mediums,[99] containing ammonium sulfate as a source of nitrogen, but some require added purines.[5]

The relative resistance of the cholera vibrio, as a gram-negative enteric form, to inhibitory substances such as bile salt, bismuth-sulfite and tellurite is taken advantage of in the preparation of a variety of selective mediums, liquid for purposes of enrichment and solid for isolation[74, 84] (see below).

Figure 119. *Vibrio cholerae;* pure culture in peptone water. Gram stain; × 1200.

The Heiberg Fermentation Types of Vibrios

SUGAR	TYPE					
	I	II	III	IV	V	VI
Sucrose	+	+	+	+	−	−
Mannose	+	−	+	−	+	−
Arabinose	−	−	+	+	−	−

Fermentation reactions are variable, and a number of carbohydrates, including dextrose, levulose, galactose, maltose, sucrose, and mannitol, may be fermented with the production of acid but no gas. Lactose, inulin, and dulcitol are not attacked. Heiberg studied the fermentation reactions of the vibrios at some length and arrived at six fermentative types on the basis of the fermentation of sucrose, arabinose, and mannose which are known as the Heiberg types. Type I is characterized by the fermentation of sucrose and mannose but not arabinose,

Figure 120. Flagella of vibrios; *left, Vibrio cholerae; right,* a paracholera vibrio. × 2000. (Kral.)

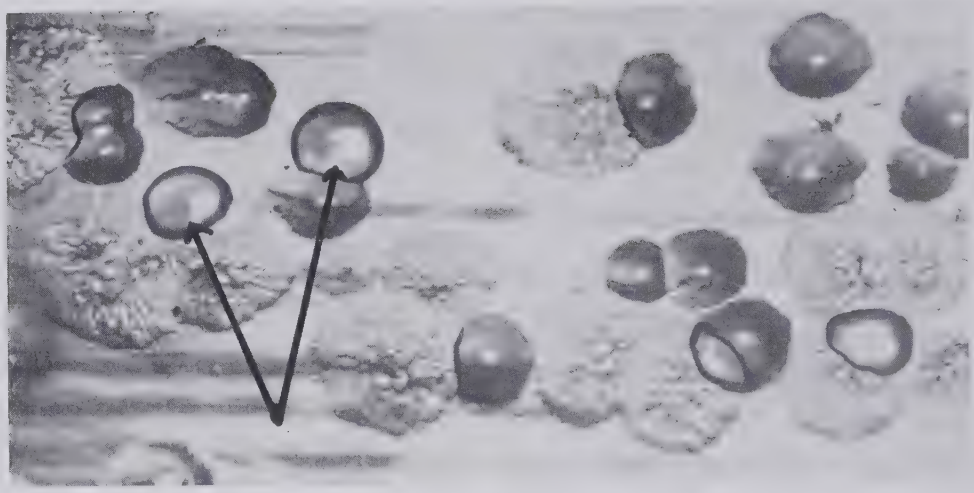


Figure 121. The colonial morphology of *Vibrio cholerae* on bile salt agar in mixed culture with coliform bacilli. The minute, translucent, raised colonies of the cholera vibrio contrast with the roughened appearance of coliform bacillus colonies in the presence of taurocholate. × 10.

and contains all of the cholera vibrios and some noncholera varieties. Starch is hydrolyzed.

Both coagulated serum and gelatin are liquefied. Stab cultures in gelatin often develop a small turnip-shaped area of liquefaction at the surface, which by evaporation of the fluid leaves a bubble-like depression, while in some growth little or no liquefaction occurs along the needle puncture. Other vibrios besides the cholera vibrio, however, produce this same type of liquefaction. Growth in milk does not produce any visible change for some time, but a slow peptonization without coagulation appears on continued incubation. Hydrogen sulfide and indol are produced, and nitrates are reduced to nitrites. The addition of sulfuric acid to a culture of the cholera vibrio in nitrate-peptone broth results in the development of a red color—the so-called cholera-red reaction. This is the nitroso-indol reaction and is given by any bacterium, as the colon bacillus for example, that both reduces nitrate and produces indol. Other vibrios give this reaction also.

The question of the hemolytic activity of the cholera vibrio has been of considerable importance in relation to its differentiation from the closely related El Tor vibrios. The term hemolysis, when applied to the vibrios, refers to hemolytic activity as determined by the Greig test, the hemolysis of erythrocytes in suspension mixed and incubated with a broth culture of the vibrios. By this test the cholera vibrio is nonhemolytic. This test is, to a considerable degree, empirical, for the question of the hemolytic activity of the cholera vibrios is a complex one.[68] The cholera vibrio is, for example, hemolytic in this kind of test when human red cells are used; hemolytic activity requires calcium which may need to be supplied, is inhibited by sodium chloride, etc.[25] Nevertheless, the Greig test has given consistent results for some five decades, and only recently its validity has been questioned in connection with the pathogenicity of certain of the El Tor vibrios (see below).

As indicated above, however, some strains of the cholera vibrio are hemolytic on blood agar. The apparent contradiction is resolved by the demonstration that, in the case of this bacterium at least, blood plate hemolysis is a hemodigestive process and basically different from the liberation of hemoglobin from suspensions of erythrocytes.

The resistance of the cholera vibrio to various injurious influences is not great. It is killed by moderately high temperatures (10 minutes at 55° C.) and is destroyed quickly by chemical disinfectants. It is particularly sensitive to dry-

ing; if a drop of broth culture be dried on a slide, the vibrios are all dead in about 2 hours. It does not survive long in association with the ordinary saprophytic bacilli of soil and water, and whether it is able to multiply outside the body in impure water is uncertain. Upon the surface of vegetables and fruits kept in a cool, moist place, the vibrios may remain viable for from 4 to 7 days. The slight resistance of the cholera vibrio, especially its sensitiveness to drying, explains the rapid and complete disappearance of cholera in once infected localities and also the circumstance that the disease is rarely, if ever, airborne.

The biochemical structure of the vibrios has been studied at some length by Linton and his co-workers.[67] They have described three types of polysaccharide and two types of protein present in the cholera and related vibrios. The polysaccharides were differentiated on the basis of their hydrolysis products; that designated type I was made up of galactose and an aldobionic acid (galactose and glucuronic acid), type II of arabinose and aldobionic acid, and type III was a polymer of glucose. The two protein types, both globulins, were differentiated on the basis of racemization, *i.e.*, optical activity of the hydrolyzed protein before and after alkali racemization. These substances occurred in combinations to give six groups of vibrios, the cholera vibrios falling into groups I and II which contained the same protein but polysaccharide types I and II respectively. The significance of these observations is by no means clear. It is evident that the polysaccharide bears no relation to the immunological character of the vibrios for the distribution of these chemically different polysaccharides is unrelated to the distribution of serotype antigens. Apparently neither the protein nor polysaccharides fractions are associated with the endotoxin.

Toxin. The evidences of profound toxemia in human infections with the cholera vibrio are strongly suggestive of the formation of toxic substances by this microorganism. It was early shown by Pfeiffer in 1892 that, while filtrates of young broth cultures were only slightly toxic, vibrios killed with heat or chloroform from young agar cultures were markedly toxic to guinea pigs on intraperitoneal inoculation. The toxicity of cell-free filtrates of old broth cultures led a number of the earlier investigators to the conclusion that a soluble exotoxin is produced. It is now established, however, that no exotoxin is formed, and the toxicity of old broth cultures is attributable to dissolution of the vibrios and the liberation of endotoxin. Toxicity is variable to a degree from one strain to another,[41] and under experimental conditions the vibrios are more highly toxigenic when grown at room temperature than at body temperature or higher.[43]

Nature. The endotoxin is heat-stable, and it was found by Metchnikoff to be diffusible through collodion sacs. The isolation of a toxic protein has been reported by some workers, and the toxicity has been extracted by the trichloracetic acid method. On the basis of the latter observations it has been assumed that the toxin is a polysaccharide-lipid complex similar to those isolated from typhoid and dysentery bacilli. Gallut and his co-workers[45] have shown that the endotoxicity consists of two substances, an antigenic thermostable glucolipid and a nonantigenic thermolabile protein-like substance. Two toxic activities, having different chemical properties, have also been found by Freter,[36] one of which appears to be identical with the acid-soluble "4-hour toxin" of Gallut. It has also been reported that the cholera toxin is associated, and possibly identical, with a dialyzable phospholipid which may be extracted from the intact vibrios with organic solvents such as alcohol, ether, and chloroform.[8] Still others have reported the isolation of heat-labile toxic protein from cholera and related vibrios.[58]

The nature of the cholera toxin, or toxins, thus as yet eludes precise definition. Available evidence suggests that if it exists as a single substance in the bacterial cell, it is labile in that the biochemical properties associated with toxicity are variable and a consequence of

the methods of extraction and purification used. Or the toxin may be multiple in nature, yielding various toxic components on fractionation. In any case, it does not appear to be closely analogous to the conventional endotoxin of the gram-negative bacilli beyond its extraction as a Boivin-type substance.

Permeability effect. Toxicity as deduced from lethal effects on experimental animals, such as mice, is a questionable concept. As described elsewhere (Chap. 9), the endotoxins do not have characteristic pharmacological activity other than producing changes in permeability which undoubtedly has a complex basis, *e.g.*, relationship to adrenal hormones, the Shwartzman reaction, etc. In cholera the localization of the infection in the lumen of the bowel (see below) suggests the primary importance of toxicity in the pathogenesis of the disease, to produce a profound alteration in bowel permeability.

Such activity has been demonstrated both *in vivo* and *in vitro*. As regards the former, De and his co-workers have shown that intravenous inoculation produces changes in the bowel in the rabbit,[28] and that direct inoculation into ligated sections of the small bowel of culture[26] or cell-free culture filtrate[23] results in the secretion of large amounts of fluid into the lumen and congestion and edema of the wall of the loop. This gut reaction has been applied by others, not only to cholera vibrios, but also to enteropathogenic coliforms as noted elsewhere (Chap. 20). Toxicity manifested in this way may well play a significant part in the pathogenesis of the disease.[31]

It has also been possible to show related effects *in vitro*. It has been found[17] that the vibrio toxicity produces an increased permeability of living membranes, including sections of the small intestine of the guinea pig, as an increased flow of fluid. More precise studies[37] have shown that the toxicity markedly inhibits the sodium pump in the frog skin, and rice-water stools from cholera patients have the same activity.[54] If active sodium transport across the intestinal mucosa were decreased or abolished, excretion of large amounts of water and sodium from the intestine—diarrhea—would be expected to occur.

Mucinase. The cholera vibrio and related forms also produce mucinase activity which desquamates the intestinal epithelium of the guinea pig *in vitro* and is titrated *in vitro* against ovomucin.[20, 60] More than one serologically different mucinase is produced by *V. cholerae,* and potent mucinases are produced by noncholera vibrios, so that the relation of this activity to the pathogenesis of the disease is doubtful.[34]

Antigenic Structure. The first demonstration of bacterial agglutination with homologous immune serum by Gruber and Durham in 1896 was the agglutination of the cholera vibrio and the typhoid bacillus. It was soon apparent that the agglutination reaction was specific, and it has been used for the identification of the cholera vibrio since. The antigenic structure of the cholera and related vibrios has, therefore, been of considerable interest in connection with the differentiation of *V. cholerae.*

The vibrios contain both somatic, heatstable O antigens and flagellar, heatlabile H antigens. The type-specific antigens are O antigens, and a number of serological groups have been established; the cholera vibrio and certain of the El Tor vibrios (see below) fall into that designated O group I.

It was first shown by the Japanese workers that three serological types of the cholera vibrio may be differentiated on the basis of the specificity of the heatstable O antigens. These are: the "original," J, or Inaba type; the Hikojima, "middle" or "intermediate" type; and the "variant," F, or Ogawa type. For many years only the Inaba type was found in India, but now the Ogawa type is often observed. The Hikojima type appears to occur primarily in China together with the Ogawa type and Inaba type. Some have questioned the validity of the Hikojima type, but there is no doubt that it occurs.[40] The distribution of these types has no epidemiological significance, at least in India, and, in fact, their stability is uncertain. Shrivastava

Serological Types of Cholera and Paracholera Vibrios

JAPANESE TYPE		IMMUNOLOGICAL TYPE
NAME	SYNONYMS	
None	None	Type A
Inaba	"J," Japonica 1911, "original," "end type"	Type AC
Ogawa	"F," Formosicana 1911, "variant," "end type"	Type AB
Hikojima	"Middle type"	Type ABC

and White[93] found that cultivation in the presence of heterologous antiserum results in conversion of Ogawa strains to Inaba, and of some Inaba strains to Ogawa. Other workers[4] have found, however, that the change seems to occur in only one direction, *i.e.*, cultures of the Ogawa serotype tend to throw off Inaba variants. In this connection it is of interest that during epidemic cholera the earlier isolates tend to be Ogawa, with the Inaba serotype appearing more often later in the epidemic.[92]

A detailed analysis of the O and H antigenic structure of the cholera and related vibrios showed that a group-specific O antigen, designated A, is shared by all vibrios of group I, and that the Japanese types are determined by subsidiary O antigens, B and C, and arbitrarily designated type-specific. It was suggested that the Japanese types be indicated by their antigenic formulas, *i.e.*, Inaba as type AC, Ogawa as type AB, and Hikojima as type ABC. Vibrios containing the group antigen but lacking these type-specific antigens have been found and thus constitute a new type. Additional antigens making up a variety of serotypes have been described.[13, 38, 103] The occurrence of certain antigenic types has been questioned,[62, 94] but the low titer antiserums used prevented their demonstration; type A, for example, has been observed among strains isolated in Bangkok in 1958–59. Antigens other than the group- and type-specific antigens are demonstrable also by the application of other methods of assay such as gel diffusion.[48, 49, 73] The H antigens, designated by Arabic numerals, and other components of the O antigen are shared by the O group I vibrios with vibrios of other O groups.

Antigens other than those demonstrable by the conventional agglutination-agglutinin absorption method of antigenic analysis were first observed as giving rise to protective antibody effective in the mouse and guinea pig enteric infection assay,[9, 12] and producing antibody demonstrable in the passive hemagglutination reaction[16] and by complement-fixation.[14] Gallut[44] separated this antigenic complex as a precipitating antigen by phenol extraction of specific polysaccharide-containing antigens. This complex, designated nonagglutinogen antigen, or NAA, is shared in part with noncholera vibrios.

A curious terminology has crept into the cholera literature with respect to agglutination; it is said that a strain which is agglutinated by specific antiserum is "agglutinable" while those vibrios which are not agglutinated by cholera antiserum are called "inagglutinable" despite the fact that they are readily agglutinated by homologous antiserum. These are referred to as the nonagglutinating vibrios, or NAG vibrios.

The cholera vibrios are serologically related to Brucella, and this has given rise to positive Brucella agglutination tests in persons who are not infected with Brucella but have been immunized with cholera vaccine. Gallut[39] has shown that the common antigens are the C and D antigens of the cholera O antigenic complex. An antigenic relationship of certain vibrio strains to coliform bacilli of the Bethesda group has been described also.[70]

Variation. The cholera vibrio is well known for its tendency to develop bizarre involution forms. These are found not only in old cultures and cultures grown under somewhat adverse conditions, such

as increased salt concentrations, but also are produced by the inclusion of substances such as glycine or alanine in the medium. Such changes in morphology are also associated with the usual type of S-R dissociation which occurs readily under the influence of bacteriophage, lithium chloride, etc., the rough variants showing distinctive colonial character, spontaneous agglutination in salt solution, and so on.

The immunological changes associated with the S-R dissociation have been studied in some detail by White.[104] He has found that four groups of rough variants are immunologically distinguishable, and his group A appears to correspond to the group I of Gardner and Venkatraman. Further degeneration to the so-called ρ variants results in a loss of specific O antigen. An independent type of variation, rugose-nonrugose, may be induced, and the rugose strains of S, R, and ρ variants contain an O antigen that is common to both members of group A and vibrios of other groups. A somatic protein antigen has been isolated by White which is common to all known variants and shows wide cross-precipitin reactions throughout the vibrio group, although it appears not to be concerned in the agglutination reaction.

Pathogenicity for Man.[24, 65] The causal connection between Asiatic cholera and the microorganism discovered by Koch has been demonstrated by a number of laboratory accidents. One of the first occurred in Koch's laboratory, and other infections resulting from the accidental swallowing of cultures of the cholera vibrio have been noted since. In one instance the swallowing was deliberate; Pettenkofer and Emmerich voluntarily swallowed a small quantity of broth culture of "Koch's vibrio" and as a result developed cholera.

Both laboratory cases of cholera and those cases contracted naturally in the course of epidemics are marked by great differences in the susceptibility of different individuals. This is probably a consequence in part of innate individual differences in natural resistance, and in part of predisposing factors. Fatigue, the excessive use of alcohol, and various factors leading to mild, nonspecific gastrointestinal derangements predispose in a marked degree to attacks of cholera.

The incubation period is short; it is usually given as 3 to 5 days but may occasionally be as short as 24 hours. When the vibrios have entered the small intestine and establish themselves they multiply rapidly and, with autolysis and dissolution of the cells, endotoxin is liberated and symptoms appear. There are two clinical stages, the first that of vomiting and profuse diarrhea with the characteristic rice water stools containing flakes of mucus, shed epithelial cells, and enormous numbers of the vibrios. Biochemical examination of the cholera stool has shown that the fluid portion resembles plasma minus protein and is a transudate in nature with the mucous membrane of the intestine serving as a semipermeable membrane. Extensive biopsy studies of specimens taken at intervals during the disease have shown that the mucous membrane does, in fact, remain intact.[47, 96] With the tremendous loss of fluid, dehydration and hypochloremia become marked, and collapse, the algid stage, ensues with circulatory failure, subnormal temperature, and anuria. Treatment consists of replacement of fluid electrolyte.[76] Death may occur in either the first or second stage. The case fatality rate is over 50 per cent in untreated cases and about 30 per cent in treated cases but the disease may occur in milder form, as in the Chungking epidemic in 1945, and in Bangkok in 1959.[77]

Cholera in man appears to be largely, if not entirely, a toxemia, for the infection is confined to the lumen of the intestine and vibrios are found in the organs only rarely. It is not altogether clear whether toxin is absorbed. In the opinion of many the profound dehydration and hypochloremia and consequent impairment of renal function are sufficient to cause the clinical symptoms and pathology.

Carriers. On recovery the vibrios disappear rapidly and are discharged for only a short time. In the series of 200

cases studied by Ying 98 per cent were negative by the end of the second week, only a few giving positive cultures as late as the third and fourth weeks. In a study of 113 continuously observed cases Gilmour[50] found that 71.6 per cent were negative after the first week, 89.3 per cent after 2 weeks, and 98.1 per cent after 3 weeks, with maximal periods of intermittent excretion of 20, 21, 23, and 25 days. Similar results were observed in the Egyptian epidemic of 1947, with 96 per cent of patients negative after 3 weeks with a maximal period of intermittent excretion of 39 days.

There appears to be no chronic carrier state established in cholera. The statements in the literature regarding chronic carriers refer to persons who discharge "agglutinable" vibrios of the El Tor type; a chronic carrier of typical virulent cholera vibrios has never been observed.[86] It is probable that casual carriers occur during epidemics, and such a carrier state was demonstrated by Egyptian workers during the 1947 epidemic.[51] A carrier incidence of 2 to 4 per cent in contacts was observed, but Gohar and Makkawi found that it did not persist more than 10 days. While the carrier may play some part in the spread of the disease,[82] the case, especially in the incubation period, is probably the more important source of infection.

Bacteriological Diagnosis.[3, 15] As indicated above, the vibrios are present in the rice water stools in very large numbers and can usually be isolated from fresh specimens without difficulty. At times they are present in sufficient numbers that they can be found in stained smears, preferably made from a flake of mucus. The fluorescent antibody technique has been applied to the rapid provisional identification of the vibrios.[19, 32, 72] Both enrichment and direct streaking of agar mediums are used for cultures. For enrichment a few drops of the stool are added to a tube of alkaline (pH 8.0 to 9.0) peptone water. The vibrios grow much more rapidly than the other intestinal bacteria, and after 6 to 8 hours' incubation form a thin film of growth on the surface of the medium which can be smeared and stained, and streaked on agar.

A number of agar mediums have been used for isolation of the cholera vibrio, including starch-phenolphthalein agar and the alkaline blood medium of Dieudonné. Nutrient (meat extract) agar at pH 8.0 and containing 0.5 per cent sodium taurocholate as used at the All-India Institute for Hygiene and Public Health is one of the most useful and satisfactory mediums for primary isolation. On 24 hours' incubation the cholera vibrios grow up as small to moderate size translucent smooth colonies sharply differentiable from the roughened colonies of coliform bacilli. The microorganisms are identified by slide agglutination in O group I antiserum and typed in monospecific Inaba and Ogawa antiserums if desired. With clinically typical cholera in an epidemic this is sufficient identification.

A negative Greig test, fermentation of sucrose and mannose, and failure to ferment arabinose further substantiate the identification. The Greig test is carried out in a variety of ways. The method used at the All-India Institute consists of mixing equal portions of a 24-hour broth culture of vibrios and a 5 per cent suspension of sheep erythrocytes. The mixture is incubated for 4 hours, read, stored in the refrigerator overnight, and read again.

Chemotherapy. The cholera vibrio is susceptible *in vitro* to the sulfonamides and to the antibiotics other than penicillin, and these drugs are effective against the mouse infection produced by the parenteral inoculation of vibrios suspended in mucin. In the human disease, however, chemotherapy has been completely ineffective in altering the course of the disease, though the vibrios tend to disappear from the stools somewhat more rapidly in treated cases.[64] This is perhaps to be expected in view of the pathogenesis of the disease; the vibrios are eliminated rapidly and in large numbers with the onset of symptoms, and the disease is essentially a toxemia or the consequence of a local toxic action on the bowel.

Epidemiology. As in the other enteric infections, the connecting link in the dissemination of cholera is between infected feces and the mouths of susceptible persons. In consequence, the disease is frequently water-borne and may be transmitted by any food ordinarily consumed in the raw state. The quantitative importance of contact infection is not known. Cholera differs from the other enteric diseases, however, in the highly explosive character of the outbreaks, which is attributable to the short incubation period, the high case fatality rate, and its rapid and permanent disappearance when the outbreak has subsided. The last is due in part, perhaps, to the inability of the vibrio to survive long apart from the host, but more important is the limited duration of the convalescent carrier state and the absence of chronic carriers. In a sense cholera is one of the easiest to control of the highly contagious diseases for it cannot spread when sanitary facilities, *i.e.*, sewage disposal, water supply, etc., are in efficient operation. A striking instance of this was reported in the Balkan War in 1913 in which infection was widespread in the Bulgarian Army about Sofia, but cases in the capital were largely imported and the disease showed little tendency to spread there; Sofia was efficiently sewered and had an excellent water supply.

Endemic foci. Cholera persists in inter-epidemic periods in foci of endemic infection. Endemic areas are adjacent to rivers and are low lying and densely populated. In such areas tanks and other stored water often contain cholera and related vibrios.[1, 2] Since the chronic carrier state does not occur, it is probable that the infection persists as a small number of human cases of the disease, and these occur continuously between epidemics.[21, 27] The use of phage typing in tracing sources of infection has not as yet been too productive for there is considerable overlap in sensitivity, and only a few phage strains have been used.[78, 79, 80] It is not known why this occurs in some areas but not in others.

The classic focus of endemic infection is in Bengal, extending from the Ganges and Brahmaputra deltas into Assam and Bihar. There is also a focus of infection in Burma in the Irrawaddy delta, and possibly also in parts of the Salween delta. There may also be an endemic focus of infection in China in the valley of the Yuen River, but this is not fully established.[97]

Epidemic spread. Each year the disease occurs in epidemic form, first in the endemic areas such as Bengal, beginning

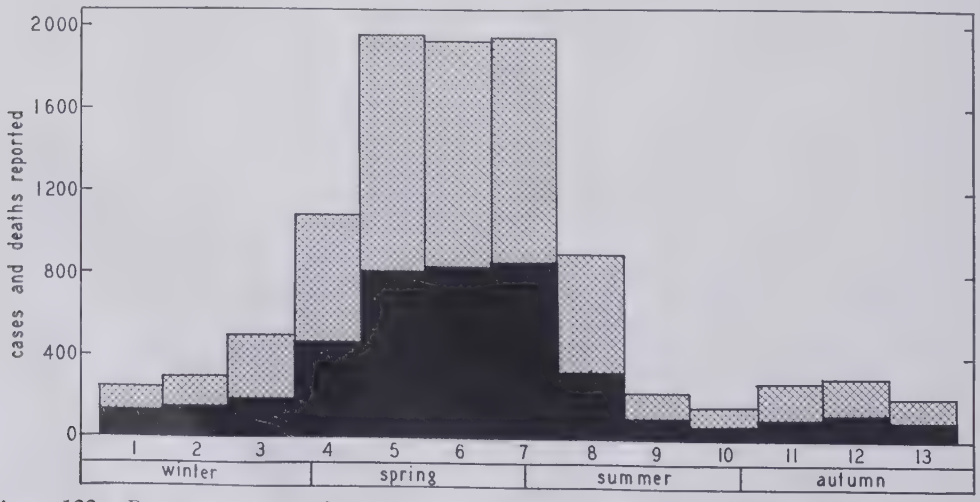


Figure 122. Representative incidence of cholera in West Bengal, shown as the mean numbers of cases and deaths reported by 4-week periods. Data from Epidemiological and Vital Statistics Reports, World Health Organization.

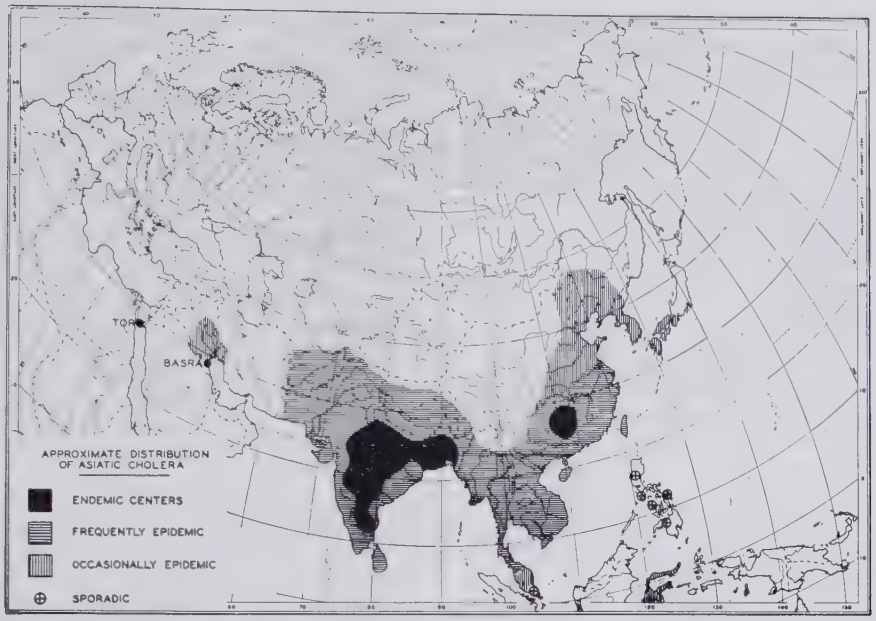


Figure 123. The approximate distribution of Asiatic cholera. The precise distribution cannot be shown because of great variability from time to time. This represents an approximate distribution during the decade 1930–1940. Based in part on map prepared by Army Medical Intelligence, epidemiological reports of the League of Nations, and various authors. (Based on Goode *Base Map* No. 205. By permission of the University of Chicago Press.)

in February. The peak is reached in April and May in Bengal just before the beginning of the monsoon season, and there is a remarkable correlation between relative humidity and the incidence of cholera.[89, 90] In Bengal a small secondary peak occurs in December, and the seasonal incidence of the disease in that area is illustrated in the accompanying figure.

The disease extends from these foci each year, reaching a peak in Punjab in July and August, and in Uttar Pradesh in the spring, and spreads through central India to Madras and Bombay. From time to time it spreads further in the Far East, appearing along the China coast, and occasionally in Manchuria, Japan, and the Philippines.

Infection spreads to the west especially via the pilgrimages; pilgrims going from the delta of the Ganges to Arabia and Mecca carry the infection and transmit it to pilgrims from Egypt and Algiers. The quarantine stations at El Tor and Basra serve to keep the disease out of Europe in normal times, but it seeps through into Iran and Iraq from time to time. With modern controls the disease is prevented from spreading as widely as at one time. There was cholera in Europe during the Balkan War and World War I, and it occurred in epidemic form in eastern Europe in 1921 and 1922, and most recently appeared in Egypt in 1947 in epidemic form. In the latter half of the nineteenth century cholera occurred in epidemic form in this country, reaching as far as the Middle West following importation at the Atlantic ports, but there has been no cholera in the United States proper since 1911. For the most part it is confined at the present time to India and adjacent areas; in the period 1948 to 1952, for example, 79 per cent of the cases occurred in India, 19 per cent in Pakistan, and 1.5 per cent in Burma, to account for 99.5 per cent of all cases.[71]

Immunity. Antibodies, agglutinins and bacteriolysins, are produced by infection with the cholera vibrio but have no diagnostic utility because of late and irregular appearance.[42] It is usually said that recovery from an attack of cholera confers an immunity to subsequent infection, but neither the quality of this

immunity nor its duration is definitely known. It seems probable that the immunity is not of a high order and that it does not persist more than a year or two at the most.

Active immunization of man with vaccines has been carried out for many years. The first vaccine was that of Haffkine (1893) who used a living attenuated culture in Spain and India. A heat-killed vaccine was used by Kolle and was reported to give good results in Japan. Sensitized vaccines, *i.e.*, vibrios treated with antiserum, have been investigated also. The cholera vaccine used at the present time in this country and in India is a saline suspension of killed vibrios, half the Inaba variety and half the Ogawa variety. Cholera vaccines are usually more concentrated, *i.e.*, are heavier suspensions, than the vaccines prepared from other enteric bacilli, and contain eight to ten thousand million vibrios per millimeter. Usually two doses of 1 ml. each are given a week apart, and a booster dose of 1 ml. every 6 months.

It is not clear how effective active immunization is. Much of the reported evidence is open to criticism and not convincing. The difficulties of carrying out an adequately controlled experiment in man are, of course, very great indeed. The earlier work was examined by Greenwood and Yule who found it unconvincing on the whole, but there was a slight presumption in favor of inoculation. Most subsequent reports have been favorable with a few definitely unfavorable. An extensive and carefully controlled field study was carried out in Madras in 1941–42, and analysis of the results indicated a definite protective effect of prophylactic inoculation, the immunes being twelve to fourteen times less likely to get the disease. This is the best evidence yet presented for the efficacy of cholera vaccination. It is generally agreed that cholera is as severe in the inoculated as in the uninoculated, and the therapeutic use of antiserums is apparently without effect.

An effective prophylactic immunity is complicated by the nature of the infection, *e.g.*, the cholera vibrio remains within the lumen of the bowel, and therefore outside the tissues, and does not come in contact with circulating antibody.[10] The occurrence of antibody within the lumen of the bowel, coproantibody, as described elsewhere (Chap. 15),[11] provides a rational basis for a prophylactic immunity preventing the establishing of a focus of infection, or the neutralization of toxicity, or both. The antibody is apparently formed locally[85, 100, 101] and, since it cannot accumulate, is transitory in contrast with the persistence of serum antibody. It has been observed that the presence of coproantibody is associated with immunity to experimental enteric infection.[18, 35, 59] It is not yet known whether or not the relatively small amounts of antibody present are sufficient to give a significant immunity to the naturally acquired disease, but if so such an immunity would persist for only a few months.

Pathogenicity for Lower Animals. Under natural conditions domestic animals and the animals used in the laboratory never contract cholera. It is possible to produce enteric infection with the cholera vibrio in the guinea pig by intragastric inoculation with very large numbers of the vibrios either suspended in alkaline buffer or following oral administration of bicarbonate, provided that intestinal motility has been reduced by prior administration of tincture of opium. A true infection is produced with active multiplication of vibrios in the intestine, but without generalized invasion of the body, and with their excretion in the feces which persists for 10 to 14 days and occasionally as long as 3 weeks in nonfatal infection.[11, 83] The suckling rabbit may be similarly infected[30, 91] and is considerably more susceptible than the guinea pig but after weaning cannot be infected. A fatal enteric infection may be produced in the guinea pig by intragastric inoculation with a streptomycin-resistant strain of *V. cholerae* suspended in streptomycin solution.[33] Apparently the streptomycin in the inoculum suppresses the growth of the normal intestinal flora, allowing it to be overgrown

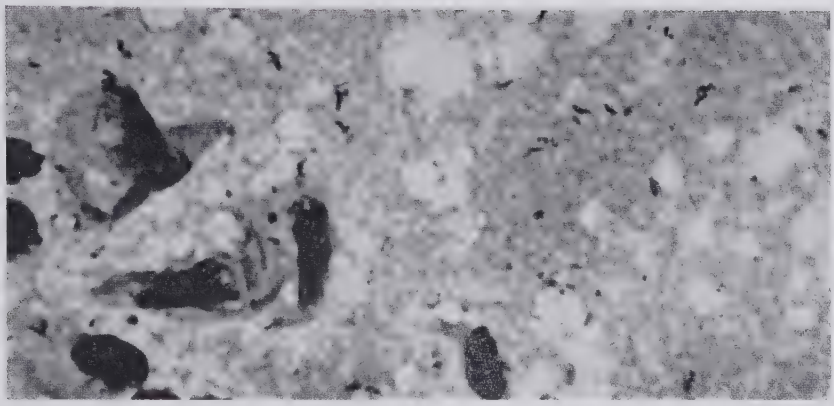


Figure 124. *Vibrio cholerae* in peritoneal exudate of a guinea pig. Note the swollen and aberrant forms. Gram stain; × 1250.

by the cholera vibrio. It is not clear how closely related these experimental infections are to human cholera.

The guinea pig is susceptible to intraperitoneal inoculation of living vibrios and "intraperitoneal cholera" has been extensively studied by the earlier workers. Griffitts[52] has shown that the virulence of the cholera vibrio for mice by intraperitoneal inoculation may be markedly enhanced by suspension in 5 per cent mucin; as few as fifty living vibrios may kill by this method. This will undoubtedly be of considerable utility in assay of virulence and active and passive immunity but would appear to be quite unrelated to the enteric infection of man. The developing chick embryo may also be infected with the cholera vibrio and is protected by the inoculation of immune serum.[105]

Active or passive immunization of experimental animals to parenteral inoculation of the cholera vibrio is highly successful, and the usual immunization procedures yield high titer antiserums. The immunity so conferred is apparently almost wholly an antibacterial immunity, for little antitoxic activity may be detected. It was shown by Pfeiffer in 1894 that lysis of the vibrios by immune serum occurs readily. The Pfeiffer phenomenon may be demonstrated in either actively or passively immunized guinea pigs by intraperitoneal inoculation of living vibrios; if peritoneal fluid is withdrawn from time to time and examined, it will be found that the vibrios lose their motility, become swollen and misshapen, and then disintegrate. The phenomenon may be produced *in vitro* with potent antiserum and complement.

PARACHOLERA VIBRIOS AND NON-CHOLERA VIBRIOS

For the most part as an adjunct to the study of the cholera vibrio, a considerable number of vibrios have been isolated from water and from the feces of individuals suffering from mild diarrheal disease, so-called cholera nostra. Some of these have been given place names such as *V danubicus*, *V. ghinda*, and *V. massauah*. A phosphorescent vibrio, *V. phosphorescens*, has been isolated from water, and *V. proteus* from human feces. All of these differ immunologically from the cholera vibrio, *i.e.*, belong to O groups other than I. Vibrios of other than O group I and Heiberg type I are occasionally found in diarrheal disease. Casual association is probable in sporadic cases for water vibrios are often present

in great numbers in drinking water in cholera areas, and may be found from time to time in fecal specimens. On occasion such vibrios are found associated with epidemic gastroenteritis, presumably in causal relation to it.[53]

The El Tor Vibrios. The best known of the so-called paracholera vibrios are those which were first isolated at the Tor quarantine station in 1906 from pilgrims suffering from diarrheal disease. A considerable number of strains were isolated at Tor in 1930 and 1931 by Doorenbos, and apparently these vibrios can be isolated with some frequency there. Very many, though not all, of the El Tor strains are "agglutinable," that is to say, agglutinate with anticholera serums, and have been shown to belong to O group I. These, like the cholera vibrio, contain both group- and type-specific O antigens, giving rise to El Tor vibrios of the Inaba or Ogawa types. The agglutinable strains differ from the cholera vibrio only in that they are actively hemolytic as noted earlier; a number of physiological tests, such as the Voges-Proskauer reaction, have been said to differentiate the two, but none has proved to be reliable.

The relationship of these vibrios to Asiatic cholera has been a matter of considerable interest; aside from general considerations, their presence raises a specific practical question at quarantine stations. Doorenbos is strongly of the opinion that two types of cholera may be distinguished, the epidemic form caused by the classic cholera vibrio and the endemic form caused by atypical variants of *V. cholerae* such as the El Tor vibrio; inherent in this is the assumption that the El Tor vibrios may become typical *V. cholerae*. This view is not generally accepted.

The Celebes vibrio. The question of the pathogenicity of the hemolytic agglutinable vibrios was raised again by the occurrence of an epidemic, apparently of cholera, in Celebes in 1937 and 1938.[69, 75, 102] About 400 strains of the vibrio were isolated and all proved to be hemolytic and agglutinable with cholera antiserum. According to de Moor, they are differentiated from the cholera vibrio by agglutination in O group I antiserum diluted with 0.3 per cent sodium carbonate, while the cholera vibrio is not, and by failure to precipitate when equal volumes of 0.5 per cent mercuric chloride and vibrios suspended in 0.5 per cent sodium bicarbonate are mixed. This epidemic, in which many cases were clinically indistinguishable from cholera though some were mild, is convincing evidence of a high degree of pathogenicity of at least some strains of the hemolytic agglutinable vibrios.

Since 1940 such disease, sometimes called choleriform enteritis, has reappeared in Indonesia from time to time [46, 98] where it appears to be endemic, and has also occurred in India.[81] It has subsequently spread in pandemic form in Southeast Asia, occurring in Thailand, Laos and Cambodia in 1958 and recurring in 1959 and 1960. In 1961 it appeared in Kwantung Province, and in Sarawak, Macao and Hongkong. It spread to the Philippines in 1961-62 where it continues to recur. As late as 1958 the WHO Committee on International Quarantine excluded "paracholera El Tor" or "enteritis choleriformis El Tor" from the definition of quarantinable disease. Nevertheless, the differentiation of this disease from cholera is of no practical significance in relation to treatment and control,[87] and in 1962 the Committee ruled that the disease is not to be distinguished from cholera under international quarantine regulations and cholera vaccine is applicable for purposes of immunization.[88] It is not yet clear whether there is some interrelationship between the classic nonhemolytic cholera vibrio and the pathogenic El Tor strains as suggested by Doorenbos (see above), whether classic cholera may be caused by a group of pathogenic vibrios rather than a single sharply defined species, or whether the appearance of cholera of El Tor etiology represents a new etiological entity.

Other Pathogenic Vibrios. A number of other vibrios are known which pro-

duce disease in animals but which are apparently not pathogenic for man. *Vibrio metchnikovii* was isolated in 1888 from fowls suffering from an epidemic disease resembling fowl cholera. It closely resembles the cholera vibrio morphologically and physiologically and is highly pathogenic for guinea pigs and pigeons while the cholera vibrio is not pathogenic for the latter. It differs from *V. cholerae* immunologically and is neither agglutinated nor lysed by anti-cholera serum.

An epidemic disease of carp and other fish caused by a vibrio designated *V. piscium* has been described by David.[22] The vibrio resembled the cholera vibrio morphologically and was immunologically related to it.

Vibriosis of domestic animals. A microorganism designated *Vibrio fetus* infects cattle, sheep, and goats to produce abortion in pregnant females and an essentially symptomless infection in males. The vibrio differs sharply from the cholera and paracholera vibrios in that it requires enriched mediums for growth and is relatively inactive biochemically,[66] and is antigenically distinct, falling into a number of serotypes.[6] The naturally occurring disease is closely reproduced in the pregnant female guinea pig. The infection is transmitted by contact, and in cattle[7, 55] and goats, but apparently not in sheep,[61] the infected male acts as a carrier to spread the infection by breeding. In the female, agglutinating antibody occurs in the cervico-vaginal secretions to considerable titer and is considered to be diagnostically reliable in contrast to irregular serum agglutinin. A few human infections have been reported[63, 95] some of which resemble brucellosis; the infection is a generalized one and the vibrios are found in the blood.

Two other vibrio species are responsible for diarrheal disease of certain domestic animals. *Vibrio jejuni* infects calves; it differs from *V. fetus* culturally and serologically and is nonpathogenic for guinea pigs. *Vibrio coli* is the etiological agent of swine dysentery.[29]

REFERENCES

1. Abou-Gareeb, A. H.: Cholera vibrios in Calcutta mosque water. J. Trop. Med. Hyg., 1959, **62:**195–197.
2. Abou-Gareeb, A. H.: Experimental study of cholera vibrio strains in tank waters. J. Trop. Med. Hyg., 1960, **63:**192–194.
3. Ahuja, M. L., Krishnan, K. V., Pandit, S. R., and Venkatraman, K. V.: Laboratory diagnosis of cholera. A note on bacteriological procedures. Indian J. Med. Res., 1951, **39:** 135–139.
4. Bhaskaran, K., and Gorrill, R. H.: A study of antigenic variation in *Vibrio cholerae*. J. Gen. Microbiol., 1957, **16:**721–729.
5. Bhaskaran, K., and Rowley, D.: Nutritional studies on *Vibrio cholerae*. J. Gen. Microbiol., 1956, **15:**417–422.
6. Biberstein, E. L.: Serological variants among strains of *Vibrio fetus*. Cornell Vet., 1956, **46:**144–165.
7. Blobel, H., Simon, J., and McNutt, S. H.: Observations on experimental bovine *Vibrio fetus* infection. Amer. J. Vet. Res., 1957, **18:** 579–583.
8. Burrows, W.: The endotoxin of the cholera vibrio: Isolation and properties. Proc. Soc. Exptl. Biol. Med., 1944, **57:**306–308.
9. Burrows, W.: Studies on immunity to Asiatic cholera. VI. Observations on the relation of antibody to effective immunity to experimental enteric cholera, with a note on sulfonamide therapy. J. Infect. Dis., 1953, **92:** 152–163.
10. Burrows, W.: Effective immunity to enteric infection with special reference to cholera. University of Maryland Lectures on Theoretical and Applied Aspects of Modern Microbiology. 1961/62.
11. Burrows, W., Elliott, M. E., and Havens, I.: Studies on immunity to Asiatic cholera. IV. The excretion of coproantibody in experimental enteric cholera in the guinea pig. J. Infect. Dis., 1947, **81:**261–281.
12. Burrows, W., Mather, A. N., Elliott, M. E., and Havens, I.: Studies on immunity to Asiatic cholera. III. The mouse protection test. J. Infect. Dis., 1947, **81:**157–174.
13. Burrows, W., Mather, A. N., McGann, V. G., and Wagner, S. M.: Studies on immunity to Asiatic cholera. II. The O and H antigenic structure of the cholera and related vibrios. J. Infect. Dis., 1946, **79:**168–197.
14. Burrows, W., Musteikis, G. M., and Danziger, C.: Studies on immunity to Asiatic cholera. XI. The distribution of complement-fixing antigen in electrophoretic fractions of heat-stable cell wall and intracellular substance of *Vibrio cholerae* serotypes. J. Infect. Dis., 1961, **109:**172–182.
15. Burrows, W., and Pollitzer, R.: Laboratory diagnosis of cholera. Bull. W. H. O., 1958, **18:** 275–290.
16. Burrows, W., and Schlub, L. J.: Studies on immunity to Asiatic cholera. X. Heat stable cell wall and intracellular antigens of *Vibrio cholerae* independent of agglutinogens and demonstrable by passive hemagglutination. J. Infect. Dis., 1958, **102:**94–101.
17. Burrows, W., Wagner, S. M., and Mather, A. N.: The endotoxin of the cholera vibrio:

Action on living semipermeable membranes. Proc. Soc. Exptl. Biol. Med., 1944, **57:**311–314.
18. Burrows, W., and Ware, L. L.: Studies on immunity to Asiatic cholera. VII. Prophylactic immunity to experimental enteric cholera. J. Infect. Dis., 1953, **92:**164–174.
19. Chibrikova, E. V., Kuznetsova, V. I., Bazunova, L. P., and Dudkova, V. K.: The accelerated detection of *Vibrio comma* in water and washings from objects in the surroundings by means of fluorescence microscopy. J. Microbiol. Epidemiol. Immunobiol., 1958, **29:** 1591–1595. (Translated from the Russian.)
20. Chugh, M. L., and Jensen, K. E., and Kendrick, P. L.: Antigenic relationships and toxicity of mucinolytic preparations from *Vibrio comma* and related vibrios. J. Bact., 1956, **71:**522–527.
21. Cockburn, T. A., and Cassanos, J. G.: Epidemiology of endemic cholera. Pub. Health Repts., 1960, **75:**791–803.
22. David, H.: Über eine durch choleraähnliche Vibrionen hervorgerufene Fischsenche. Zent. f. Bakt., I Abt., Orig., 1927, **102:**46–60.
23. De, S. N.: Enterotoxicity of bacteria-free culture-filtrate of *Vibrio cholerae*. Nature, 1959, **183:**1533–1534.
24. De, S. N.: Cholera: Its Pathology and Pathogenesis. Oliver and Boyd, Edinburgh and London, 1961.
25. De, S. N., Bhattacharyya, K., and Roychaudhury, P. K.: The haemolytic activities of *Vibrio cholerae* and related vibrios. J. Path. Bact., 1954, **67:**117–127.
26. De, S. N., and Chatterjee, D. N.: An experimental study of action of *Vibrio cholerae* on the intestinal mucous membrane. J. Path. Bact., 1953, **66:**559–562.
27. De, S. N., Lahiri Choudhury, P. K., and Mondal, A.: Observations on epidemic and non-epidemic cholera in Calcutta. Trans. Roy. Soc. Trop. Med. Hyg., 1958, **52:**349–353.
28. De, S. N., Sarkar, J. K., and Tribedi, B. P.: An experimental study of the action of cholera toxin. J. Path. Bact., 1951, **63:**707–717.
29. Doyle, L. P.: The etiology of swine dysentery. Amer. J. Vet. Res., 1948, **9:**50–51.
30. Dutta, N. K., and Habbu, M. K.: Experimental cholera in infant rabbits: A method for chemotherapeutic investigation. Brit. J. Pharmacol., 1955, **10:**153–159.
31. Dutta, N. K., Panse, M. V., and Kulkarni, D. R.: Role of cholera toxin in experimental cholera. J. Bact., 1959, **78:**594–595.
32. Finkelstein, R. A., and Labrec, E. H.: Rapid identification of cholera vibrios with fluorescent antibody. J. Bact., 1959, **78:**886–891.
33. Freter, R.: The fatal enteric cholera infection in the guinea pig, achieved by inhibition of normal enteric flora. J. Infect. Dis., 1955, **97:** 57–65.
34. Freter, R.: The serologic character of cholera vibrio mucinase. J. Infect. Dis., 1955, **97:**238–245.
35. Freter, R.: Coproantibody and bacterial antagonism as protective factors in experimental enteric cholera. J. Exptl. Med., 1956, **104:** 419–426.
36. Freter, R.: Two different toxic fractions extracted from *Vibrio cholerae*. J. Infect. Dis., 1956, **99:**207–211.
37. Fuhrman, G. J., and Fuhrman, F. A.: Inhibition of active sodium transport by cholera toxin. Nature, 1960, **188:**71–72.
38. Gallut, J.: Contribution à l'étude de l'antigène thermostable du vibrion cholérique. Applications practique de l'analyse antigènique O. Ann. Inst. Pasteur, 1949, **76:**122–136.
39. Gallut, J.: Relations antigèniques entre vibrion cholérique et brucelles. Ann. Inst. Pasteur, 1950, **79:**335–338.
40. Gallut, J.: Sur le type Hikojima du vibrion cholérique. Ann. Inst. Pasteur, 1953, **84:**428–430.
41. Gallut, J.: Contribution à l'étude de la toxine cholérique. Variation du pouvoir toxique de *Vibrio cholerae* (Ogawa) au cours de la maladie. Ann. Inst. Pasteur, 1954, **86:**561–569.
42. Gallut, J.: La seroagglutination dans le diagnostic retrospectif du cholera. Bull. Soc. Path. Exot., 1954, **47:**657–663.
43. Gallut, J.: Contribution à l'étude de la toxine cholérique. Influence de la temperature d'incubation sur le pouvoir toxigène in vitro de *Vibrio cholerae* (Inaba). Ann. Inst. Pasteur, 1955, **89:**242–244.
44. Gallut, J.: Contribution à l'étude du complexe antigènique "O" des vibrions. Relations immunologiques entre *V. cholerae* et vibrions dits inagglutinables (NAG). Ann. Inst. Pasteur, 1960, **99:**28–55.
45. Gallut, J., and Graybar, P.: Recherches immunochemiques sur le vibrion cholérique. III. Mise en evidence de deux constituents toxique de nature differente dans la toxine cholérique. Ann. Inst. Pasteur, 1945, **71:**83–92.
46. Gan, K. H., Achja, E. M., Sukono, R., and Warsa, R.: First cases of paracholera El-Tor (enteritis choleriformis El Tor van Loghem) in Java. Trop. Geograph. Med., 1958, **10:**113–116.
47. Gangarosa, E. J., et al.: The nature of the gastrointestinal lesion in Asiatic cholera and its relation to pathogenesis: A biopsy study. Amer. J. Trop. Med. Hyg., 1960, **9:**125–135.
48. Ghosh, S. N., and Mukerjee, S.: Studies on antigens of *Vibrio cholerae* by gel diffusion technique. Part I. Observations on the antigenic make-up of soluble vibrio material. Ann. Biochem. Exptl. Med., 1960, **20:**31–36.
49. Ghosh, S. N., and Mukerjee, S.: Studies on antigens of *Vibrio cholerae* by gel diffusion technique. Part II. Further observations on the antigenic analysis of materials soluble in water. Ann. Biochem. Exptl. Med., 1961, **21:**151–156.
50. Gilmour, C. C. B.: Period of excretion of *Vibrio cholerae* in convalescents. Bull. W. H. O., 1952, **7:**343–351.
51. Gohar, M. A., et al.: Some observations on the carrier state in cholera. J. Trop. Med. Hyg., 1952, **55:**241–245.
52. Griffitts, J. J.: The use of mucin in experimental infections of mice with *Vibrio cholerae*. Pub. Health Repts., 1942, **57:**707–710.
53. Gupta, N. P., et al.: Investigations into the nature of the vibrio strains isolated from the epidemic of gastro-enteritis at Kumbh Fair at Allahabad in 1954. Indian J. Med. Sci., 1956, **10:**781–791.
54. Huber, G. S., and Phillips, R. A.: Cholera and the sodium pump. Research Report MR

005.09-1040.1.7. Bureau of Medicine and Surgery, Department of the Navy, Washington, D. C. 1960.
55. Hughes, D. E.: Notes on vibriosis, with special reference to the isolation of *Vibrio fetus* from semen and preputial fluids. Cornell Vet., 1956, **46:**249–256.
56. Jenkin, C. R.: The immunological relationship of a protein toxin from *Vibrio cholerae* to its 'O' somatic antigen. Immunol., 1959, **2:**363–367.
57. Jenkin, C. R., and Rowley, D.: Possible factors in the pathogenesis of cholera. Brit. J. Exptl. Path., 1959, **40:**474–481.
58. Jenkin, C. R., and Rowley, D.: Toxic proteins from *Vibrio cholerae* and water vibrios which are lethal for mice. J. Gen. Microbiol., 1959, **21:**191–202.
59. Jenkin, C. R., and Rowley, D.: The importance of antibody in the prevention of experimental cholera in rabbits. Brit. J. Exptl. Path., 1960, **41:**24–30.
60. Jensen, K. E.: Immunologic characterization of a mucinolytic enzyme of *Vibrio cholerae*. J. Infect. Dis., 1953, **93:**107–110.
61. Jensen, R., Miller, J. A., and Hammarlund, M. A.: Vibrionic abortion in sheep. I. Transmission and immunity. Amer. J. Vet. Res., 1957, **18:**326–329.
62. Kauffmann, F.: On the serology of the *Vibrio cholerae*. Acta Path. Microbiol. Scand., 1950, **27:**283–299.
63. King, E. O.: Human infections with *Vibrio fetus* and a closely related vibrio. J. Infect. Dis., 1957, **101:**119–128.
64. Lahiri, S. C.: Chemotherapy in cholera. Brit. Med. J., 1951, **i:**500–504.
65. Lankford, C. E.: Factors of virulence of *Vibrio cholerae*. Ann. N. Y. Acad. Sci., 1960, **88:** 1203–1211.
66. Lecce, J. G.: Some biochemical characteristics of *Vibrio fetus* and other related vibrios isolated from animals. J. Bact., 1958, **76:**312–316.
67. Linton, R. W.: The chemistry and serology of the vibrios. Bact. Rev., 1940, **4:**261–319.
68. Liu, P. V.: Studies on the hemolysin of *Vibrio cholerae*. J. Infect. Dis., 1959, **104:**238–252.
69. van Loghem, J. J.: A pathogenic "El Tor" vibrio from the Netherlands Indies. Bull. Office Internat. d'Hyg. Publique, 1938, **30:** 1520–1523.
70. Malizia, W. F.: Antigenic relationship between species of Paracolobactrum and *Vibrio comma*. U. S. Armed Forces Med. J., 1954, **5:**1528–1530.
71. May, J. M.: Map of the world distribution of cholera. Geographical Rev., 1951, **41:**272–273.
72. Mikhailov, I. F., and Li Li.: Detection of *Vibrio comma* on objects of the external environment by means of fluorescent antisera. J. Microbiol. Epidemiol. Immunobiol., 1959, **30:**33–40. (Translated from the Russian.)
73. Misra, S. B., and Shrivastava, D. L.: Studies in immunochemistry of *Vibrio cholerae*. Part IV. Cross precipitin reactions of antigens in various serotypes. Indian J. Med. Res., 1961, **49:**183–188.
74. Monsur, K. A.: A highly selective gelatin-taurocholate-tellurite medium for the isolation of *Vibrio cholerae*. Trans. Roy. Soc. Trop. Med. Hyg., 1961, **55:**440–442.
75. de Moor, C. E.: An El Tor vibrio as a cause of a cholera epidemic in Celebes. Bull. Office Internat. d'Hyg. Publique, 1938, **30:**1510–1519.
76. Morgan, F. M., et al.: Treatment of cholera. J. Med. Assn. Thailand, 1959, **42:**413–422.
77. Morgan, F. M., et al.: A study of patients with mild cholera in Bangkok, Thailand, Spring, 1959. Amer. J. Hyg., 1960, **72:**250–260.
78. Mukerjee, S.: Diagnostic use of cholera bacteriophages. J. Hyg., 1961, **59:**109–115.
79. Mukerjee, S., Guha, D. K., and Roy, U. K. G.: Studies on typing of cholera by bacteriophage. Part I. Phage typing of *Vibrio cholerae* from Calcutta epidemics. Ann. Biochem. Exptl. Med., 1957, **17:**161–176.
80. Mukerjee, S., Guha Roy, U. K., and Rudra, B. C.: Studies on typing of cholera vibrios by bacteriophage. Part III. Phage typing of strains from Calcutta epidemics of 1959. Ann. Biochem. Exptl. Med., 1960, **20:**181–188.
81. Mukherji, A.: Hemolytic vibrios in cholera epidemic at Lucknow in 1945. Indian J. Med. Sci., 1955, **9:**540–543.
82. Nikonov, A. G.: Concerning the pathogenesis and diagnosis of the persistent carrier state in persons recovering from cholera. J. Microbiol. Epidemiol. Immunobiol., 1958, **29:**40–44. (Translated from the Russian.)
83. Nikonov, A. G., Yevseyeva, V. I., Bibikova, P. D., and Bichul, K. G.: Cultivation of *Vibrio comma* in the small intestine of guinea pigs. J. Microbiol. Epidemiol. Immunobiol., 1958, **29:**1925–1927. (Translated from the Russian.)
84. Ouchterlony, O.: A selective medium for isolation of cholera vibrios. Acta Path. Microbiol. Scand., 1958, **43:**274–284.
85. Pierce, A. E.: Specific antibodies at mucous surfaces. Veterinary Rev. Annot., 1959, **5:** 17–36.
86. Pollitzer, R.: A note on the incidence and epidemiological importance of cholera carriers. Bull. W. H. O. 1952, **7:**359–369.
87. Pollitzer, R.: Cholera. W. H. O. Monograph Series No. 43. World Health Organization, Palais des Nations, Geneva. 1959.
88. Report: Committee on International Quarantine. Weekly Epidemiol. Rept. W. H. O., 1962, **37:** No. 21.
89. Rogers, L.: Thirty years' research on the control of cholera epidemics. Brit. Med. J., 1957, **ii:** 1193–1197.
90. Rogers, L.: Forecasting and control of cholera epidemics in South-East Asia and China. Lancet, 1958, **i:**151–154.
91. Sanarelli, G.: De la pathogénie du cholera. V. Le "cholera intestinal" des jeunes animaux. Ann. Inst. Pasteur, 1921, **35:**745–796.
92. Sen Gupta, P. N.: Sub-types of cholera vibrios isolated from cholera cases, Calcutta. Calcutta Med. J., 1951, **48:**65–67.
93. Shrivastava, D. L., and White, P. B.: Note on the relationship of the so-called Ogawa and Inaba types of *V. cholerae*. Indian J. Med. Res., 1947, **35:**117–130.
94. Singh, G., and Ahuja, M. L.: A note on the antigenic relationship to *V. cholerae* of the so-called 'A' type of vibrio (Burrows) and 'B' type of vibrio (Gallut). Indian J. Med. Res., 1950, **38:**317–337.
95. Spink, W. W.: Human vibriosis caused by *Vibrio fetus*. J. Amer. Med. Assn., 1957, **163:** 180–182.

96. Sprinz, H.: Morphological response of intestinal mucosa to enteric bacteria and its implication for sprue and Asiatic cholera. Fed. Proc., 1962, **21:**57–64.
97. Swaroop, S.: World distribution of cholera endemicity. Epidemiol. Vital Stat. Rept. W. H. O., 1952, **5:**569–589.
98. Tanamal, S. T. W.: Notes on paracholera in Sulawesi (Celebes). Amer. J. Trop. Med. Hyg., 1959, **8:**72–78.
99. Thind, K. S.: A synthetic medium for *Vibrio cholerae*. Indian J. Med. Res., 1961, **49:**189–193.
100. Thind, K. S.: Studies of coproantibody. Part I. Effect of cortisone acetate on coproantibody formation. Indian J. Med. Res., 1961, **49:**214–222.
101. Thind, K. S.: Studies of coproantibody, Part II. The origin and nature of coproantibody. Indian J. Med. Res., 1961, **49:**223–231.
102. de Vogel, W.: The cholera epidemic of Celebes. Bull. Office Internat. d'Hyg. Publique, 1940, **32:**556–559.
103. Wahba, A.: Les facteurs antigèniques du vibrion cholérique et leur détermination par agglutination microscopique. Ann. Inst. Pasteur, 1951, **80:**639–643.
104. White, P. B.: The serological grouping of rough vibrios. J. Hyg., 1935, **35:**347–353.
105. Wilson, A. T.: Experimental vibrio infections of developing chick embryos. J. Exptl. Med., 1946, **84:**305–322.

Chapter 24

BRUCELLA

Undulant Fever; Contagious Abortion of Cattle

In 1887 Bruce, while investigating the human disease known as Malta fever, Mediterranean fever or undulant fever, discovered a microorganism in the spleen in fatal cases of the disease which he designated *Micrococcus melitensis*. A disease of goats transmissible to man, this affection not only is common on the island of Malta, where British garrisons have been often seriously affected, but occurs also on neighboring islands and on the shores of the Mediterranean Sea, and has been occasionally reported from India, South Africa, the Philippines, and the West Indies. It was first brought to attention in the United States about 1911.

In 1897 Bang, in Denmark, isolated a microorganism responsible for contagious abortion in cattle, an affection now commonly known as Bang's disease, which he termed *Bacillus abortus*. The isolation and cultivation of this bacterium in the United States were first recorded by MacNeal and Kerr in 1910.

These two diseases, one primarily of goats and secondarily of man, and the other, one of cattle, were long studied quite independently, and apparently no connection between the two was recognized prior to the work of Evans in 1918. She demonstrated the remarkably close morphological, cultural, and serological relationship existing between these bacteria which are now recognized as being intimately related to one another.

In 1914 Traum isolated from fetuses prematurely expelled from sows a bacterium which is now known to be closely related to the bacterium of Bang's disease and that of undulant fever. Regarded as three species, these bacteria have been given the generic name of Brucella and are designated as *Brucella melitensis*, *Br. abortus*, and *Br. suis*. Infection with these bacteria is termed brucellosis.[4]

Morphology and Staining. The Brucella are small coccoid or short bacillary forms varying from 0.4 to 3.0 μ in length and from 0.4 to 0.8 μ in breadth. Some variability is noted, and both coccoid and bacillary forms may appear intermingled. There is a greater tendency to the coccobacillary form in *Br. melitensis* than in *Br. suis*, with *Br. abortus* intermediate between the two, but no distinction can be made on a morphological basis. The microorganisms usually occur singly or in pairs, and in cultures short chains may be found. The smooth forms are encapsulated but spores are not formed, and these bacteria are nonmotile.

On semisolid mediums the colonies are small, circular, convex, amorphous, smooth, glistening, and translucent. No pigment is formed, but the growth of *Br. melitensis* becomes brown in older cul-

tures, and the browning extends down into the medium. This browning is shown by some strains of *Br. abortus* also.

Brucella may be stained by the usual aniline dyes, but there is a tendency toward irregular staining and, in some cases, bipolar staining. They are gram-negative.

Physiology. The nutritive requirements of these bacteria are relatively complex, and best growth is obtained on enriched mediums such as liver infusion broth or agar. Brucella have been cultivated on amino acid synthetic mediums; some strains require nicotinamide, thiamin, and pantothenic acid while others require biotin also. A number of chemically defined or synthetic mediums have been prepared which support the growth of Brucella, one of the simplest of which is that of Gerhardt and Wilson[21] containing lactate, glycerol, asparagin (or glutamic acid or histidine), thiamin, nicotinic acid, pantothenic acid, and biotin together with inorganic salts. No growth occurs at 6° C. or 45° C., and the optimum temperature is 37° C. Neither acid nor gas is produced from carbohydrate mediums, although it may be shown that glucose is utilized to a small extent, and its inclusion generally favors growth. Nitrates are reduced, and growth in milk is accompanied only by slowly increasing alkalinity. Gelatin is not liquefied, and indol is not formed. Urea is hydrolized by *Br. suis*, but not

Figure 125. *Brucella* melitensis in pure culture. Note the coccobacillary morphology. Fuchsin; × 1050.

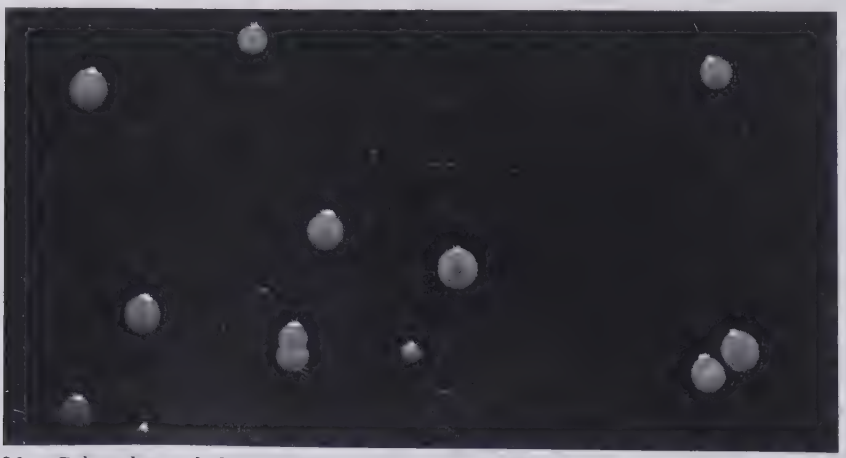


Figure 126. Colonial morphology of *Brucella melitensis* on liver infusion agar. The slight granularity is accentuated here, and the colonies are smooth, flattened and slightly brownish in color. × 4.

by *Br. abortus* or *Br. melitensis*. The optimum pH is 6.6 to 6.8.

Hydrogen sulfide. All three species produce hydrogen sulfide but differ in that *Br. suis* produces it in abundance, *Br. abortus* to a lesser extent, and *Br. melitensis* to only a slight degree. It may be noted that ammonia is produced to a greater extent by *Br. melitensis* than by the other two species.

Carbon dioxide. These bacteria are aerobic, and *Br. melitensis* and *Br. suis* may be grown on primary isolation under the usual aerobic conditions. *Br. abortus*, however, requires incubation in an atmosphere containing 10 per cent carbon dioxide on primary isolation. Subsequent transfers from the primary growth must be incubated in 10 per cent carbon dioxide, but after a number of transfers *Br. abortus* adapts itself to ordinary aerobic growth.

Dyes. The species of Brucella show a differential sensitivity to a number of dyes. Thionine and basic fuchsin may be incorporated in liver infusion agar in dilutions of 1:50,000 and 1:25,000 respectively. *Br. melitensis* and *Br. abortus* will grow in the presence of basic fuchsin, but *Br. abortus* will not, while *Br. melitensis* and *Br. suis* will grow in the presence of thionine but *Br. abortus* will not. Crystal violet and pyronine give the same reactions as basic fuchsin, and azure A and safranine O have some utility also. The growth of *Br. melitensis* is also inhibited in the presence of sodium diethyldithiocarbamate, while that of the other two species is not. Growth inhibition may be tested by the use of dye tablets analogous to the antibiotic disks used to assay antibiotic sensitivity, but higher concentrations are required than when the dye is incorporated in the culture medium; a zone of growth inhibition 4 mm. or more in diameter is taken to indicate sensitivity to the dye. The fermentation of a number of sugars, inositol, maltose, mannose, rhamnose, and trehalose, also has differential value. The physiological differentiation of Brucella species has been studied intensively by Pickett and his co-workers,[9, 41, 42] and certain of the differential reactions are summarized in the accompanying table. Investigation of metabolic patterns among the Brucella species, as the metabolism of amino acids, carbohydrates and Krebs cycle intermediates in respirometers, has shown a correlation of such pattern with species which sets *Br. suis* sharply apart—but also serves to characterize *Br. melitensis* and *Br. abortus*.[36] A phage isolated by Russian workers is effective against *Br. abortus* but not the other two species;[12, 31, 65] more precise characterization by phage typing has not yet been achieved.

Differentiation of Brucella Species

	DIFFERENTIAL REACTIONS	BR. MELITENSIS	BR. ABORTUS	BR. SUIS
sensitivity to	thionine 1:800	−	+	−
	basic fuchsin 1:200	−	−	+
	crystal violet 1:400	−	−	+
	pyronine 1:8000	−	−	+
fermentation of	inositol	−	+	−
	maltose	−	−	+
	mannose	−	+*	+
	rhamnose	−	+	−
	trehalose	−	−	+
	CO_2 required	−	+**	−
	hydrolysis of urea	− (slow)	− (slow)	+ (rapid)
	formation of H_2S	±	+	++

* usually
** on primary isolation

The status of these physiologically differentiable types, reinforced by correlated differences in antigenic character (see below), as species of the genus Brucella, and their stability, appears to be established[33, 72] although there continues to be some difference of opinion.[45, 46] The precise status is probably not of importance since Brucella species, like other species of microorganisms, have no more than the status of form

species, and the significant element is a generally accepted nomenclature.

The Brucella show the usual susceptibility to heat and disinfectants. A point of some practical importance is the rapid death of these bacteria at pasteurizing temperatures; both *Br. abortus* and *Br. suis* are killed in 3 minutes at 143° to 145° F. They persist in soil, water, and dust for 1 to 2 months but disappear within 10 days in milk, presumably in part as a consequence of the presence of acids formed by other bacteria. In this connection it is of interest that these bacteria are able to survive 2 hours or more in milk mixed with gastric juice.

Antigenic Structure. Each of the three Brucella species contains two heat-stable antigens, designated as A and M. *Br. melitensis* contains a relatively large amount of M and a small amount of A, while both *Br. abortus* and *Br. suis* contain large amounts of A and small amounts of M. The ratio of A to M is said to be about 20:1 in the case of *Br. abortus* and 1:20 in the case of *Br. melitensis*. It is possible, then, to differentiate *Br. melitensis* from *Br. abortus* and *Br. suis* by serological methods, *i.e.*, agglutination, but *Br. abortus* and *Br. suis* cannot be differentiated from one another in this way. Application of the gel-diffusion technique has shown a precipitating antigen in *Br. melitensis* which is seemingly not present in *Br. abortus*;[44] this is perhaps the M antigen. In practice monospecific serums, *i.e.*, serums adsorbed to remove the small amount of antibody common to the other immunological type, should be used.

Toxin. No exotoxin is formed by these bacteria, but the cell substance of Brucella is toxic. A toxic endo-antigen, prepared by Pennell and Huddleson[40] by tryptic digestion of dried cells followed by trichloracetic acid extraction and alcohol precipitation, appears to be a glucolipidpolypeptide complex. Further study, especially by Miles and his co-workers, has shown that the toxicity may be prepared as a highly purified material, designated aminopolyhydroxy antigen, with a mouse LD_{50} of 0.1 to 0.2 mg., and is hydrolyzed by dilute acid to phospholipid, formyl amine, and free amine, the last representing an amino sugar converted to a nonreducing anhydride during hydrolysis.[37, 38] The endotoxin appears to be substantially the same, in both nature and amount, in virulent and avirulent smooth strains. While it is considered probable that it contributes to the pathogenesis of the disease[60] and is thus a component of virulence, virulent strains appear to be able to multiply more rapidly, possibly intracellularly.[7, 18, 25] The nature of virulence is not yet clear.[48, 70]

Variation. The Brucella species dissociate relatively easily to give rise to the rough form. The environmental factors affecting the dissociative process have been studied in detail by Braun.[6] The S→R transformation is accompanied by a change to a rough colonial type, a loss of virulence, and alteration in immunological specificity. A point of particular interest is the relationship of alanine to the S→R dissociation; that produced by the S form accumulates in the culture to toxic concentrations, and the S form is replaced by the alanine-resistant R form. Other environmental factors, including oxygen tension, contribute also.[2, 3, 54] The antigenic alteration begins to take place before morphological changes are apparent and has been a source of considerable difficulty in the serological typing of these bacteria; the altered immunological types have been termed paramelitensis or para-abortus strains. It is, therefore, essential to use only smooth cultures in the serological differentiation of these bacteria.

A number of tests have been devised for the detection of antigenic variants. The rough forms are, of course, spontaneously agglutinated in saline. Slightly rough strains may be detected by their agglutination upon boiling in saline for 2 hours (thermo-agglutination test) or by slide agglutination with acriflavine. The smooth colonies may be differentiated morphologically by culture on glucose-glycerol agar for 96 hours, flooding the plate with 1:2000 aqueous crystal violet, and examining after 15 seconds by oblique transmitted light; the smooth

colonies are a light blue-green, while the nonsmooth vary from red to blue-red or violet-red.[69]

Pathogenicity for Lower Animals.[29, 49] Brucellosis is primarily a disease of domestic animals and is only secondarily communicated to man; the chief animal reservoirs are goats, cattle, and swine. It is of some interest that host specialization of the parasites has taken place, giving rise to the three species or, as some prefer to regard them, varieties of Brucella.

Goats.[66] Goats may be artificially infected with *Br. melitensis* by almost any route, and it is probable that under natural conditions the vaginal discharge at the time of aborting and shortly thereafter plays an important part in the dissemination of the infection. Agglutinins appear in the blood of artificially inoculated pregnant goats by the third or fourth day, and the titer rises rapidly to perhaps 1:1000 within 48 hours and reaches a peak of 1:2000 or thereabouts by the twelfth day of infection. Just before the peak in agglutinin titer, bacteremia is initiated which persists for perhaps one month. This acute generalized infection becomes localized during the second month after the termination of the pregnancy during which the animal was infected. In most cases the bacteria do not persist in the udder and uterus after the fifth month following termination of pregnancy. A second pregnancy does not, as a rule, cause an exacerbation of the disease, but in some cases the infection may remain localized in the area of the genital tract for several years.

The most obvious clinical symptom of infection is abortion, but it does not occur consistently. Pyrexia is apparent within 48 hours of the generalized infection, and there is slight diarrhea. The placenta is not retained, but a copious vaginal discharge is frequently observed for 2 or 3 weeks after kidding. In lactating goats the milk may be physically altered and appear in extreme cases as a clear fluid containing suspended clots.

Immature goats are highly resistant to the infection, and kids born of infected dams may not be infected and commonly do not become so in spite of the ingestion of enormous numbers of Brucella in the milk. Nonpregnant mature goats are also resistant to infection and respond to artificial inoculation with only a low and transient agglutinin titer in the blood serum.

Brucellosis in sheep is similar to that in goats.

Cattle. Brucellosis in cattle is most commonly an infection with *Br. abortus*, although both *Br. melitensis* and *Br. suis* have also been found. The microorganism may gain entrance by a variety of routes, including direct inoculation into the vagina, by way of the conjuctiva, through the unbroken skin, or via the alimentary tract. The primary symptom of the disease is abortion of the fetus by pregnant cattle. The time elapsing between initial infection and abortion varies from 3 weeks to 4 months, and the period of gestation at which abortion may take place varies from 2 to 9 months. Cattle do not abort, however, unless infected during pregnancy and even then not all abort—perhaps 30 per cent—or the cattle may become sterile. Subsequent pregnancies may proceed normally in spite of persistence of the infection; second abortions are not common, and third abortions are rare.

The bacilli may be found in the blood in perhaps 10 per cent of the cases and are very likely consistently present during the acute infection. Early in the infection the bacteria are found in the lymph glands about the head and intestines, by the end of the first month they are found all through the body, and by the end of the third month have localized in the mammary glands and are found only in the udder. The invasion of the udder results in an acute or chronic inflammation with lesions in the alveoli and interalveolar connective tissue and when the lymph glands are involved, a chronic lymphadenitis. Chronic infection of the udder may persist indefinitely without significant differences in the quality of the milk, and bacilli may be excreted over a long period, perhaps for life. The uterus, on the other hand, frees

itself of the bacteria relatively soon, and the vaginal discharges do not contain the bacilli for an extended period.

Animals infected during pregnancy show an agglutinin titer ranging from 1:200 to 1:1000 which falls slowly over a period of 6 months or so. Cattle that continue to excrete bacilli in the milk generally show persistent agglutinin titers of 1:200 or more, although a titer of 1:50 has diagnostic significance. Agglutinins are also present in the milk and may be demonstrated in the whey after clotting with rennin. Infected animals become sensitized to the bacillary substance, and a skin reaction may be elicited by the intradermal injection of a preparation of Brucella protein designated as *abortin* or *brucellergen*. As in the case of the young goats, calves are relatively resistant to the infection.

Swine.[30] Brucellosis of swine seems always to be due to infection with *Br. suis*, though these animals may be artificially infected with *Br. abortus*. In contrast with cattle, the males are commonly infected, and abortion in infected females is less frequent than in cows; about 50 per cent of swine abortions are due to unknown causes, not brucellosis. The clinical symptoms may be mild or lacking, and in a number of instances there has been no outward evidence of the disease in an infected herd, but the proportion of swine infected is as high as 20 per cent in some localities. Under natural conditions infection may take place via the alimentary tract. The infected boar, a testicular infection, is undoubtedly a significant element in the dissemination of the infection. The bacilli are eliminated with aborted fetuses and vaginal discharge, urine, semen, and milk.

Other animals. A number of other animals have been found to be naturally infected with Brucella. There is some evidence that the disease of horses known as fistula of the withers or poll evil is a Brucella infection; both *Br. abortus* and *Br. suis* have been isolated from cases of the disease. Stone[66] found that 9.5 per cent of horses tested in New York City gave positive serological reactions. Brucella infections of fowl have been reported; in one instance *Br. suis* was isolated from several naturally infected birds, but the disease is probably not common. Dogs may also be infected naturally; *Br. suis* and *Br. abortus* have been isolated. Wild rats may be artificially infected, and *Br. abortus* has been isolated from a naturally infected rat. Natural infection of rabbits with *Br. melitensis* has also been reported. Of the usual laboratory animals guinea pigs are readily infected and are most often used for experimental purposes. A fatal infection is produced in the mouse, using the mucin technique,[11] and the embryonated egg is readily infected.[20, 32]

Pathogenicity for Man.[23, 57] Man is susceptible to infection with these three species of Brucella, but infections with *Br. melitensis* and *Br. suis* are usually more severe than those with *Br. abortus*. The incubation period of undulant fever in man is highly variable and relatively long; it may range from 1 week to not less than 4 months. The case fatality is low—2 to 3 per cent. It may have varied clinical manifestations, and five types are recognized: (1) the intermittent type with shifting articular rheumatism, weakness, night sweats, and a temperature near normal in the morning but rising to 101° to 104° F. in the evening, in which the patient remains in bed in the latter part of the day; (2) the ambulatory type with much the same symptoms but to a mild degree; (3) the undulant type, generally melitensis infections, characterized by step-like increases in the temperature from day to day to a maximum and, after a time, gradual decrease in temperature and possibly successive repetitions of this sequence of events; (4) the malignant type, almost always melitensis infections, in which the temperature is high and sustained with extreme hyperpyrexia before death; and (5) an atypical chronic type which may take the form of muscular stiffness, gastric disturbances, and various neurological symptoms. In general, undulant fever is a disease of relatively long duration, 1 to 4 months, and relapses during convalescence are not infrequent. In a chronic form undulant

fever may be difficult to diagnose; or it may be effectively subclinical as indicated by the isolation of Brucella from apparently healthy persons.[34] A group of laboratory infections provided a unique opportunity for study of the evolution of the disease in man[8, 67]; an interesting feature was the association of neurosis and emotional disturbance with the prolonged convalescence.

Brucellosis in man is a generalized infection as a rule, while in lower animals it is a localized infection, particularly in cattle. The bacilli may be isolated from the blood stream in man,[19] and the development of agglutinins is a diagnostic aid. Man also becomes sensitized to the cell substance of the bacteria, a hypersensitivity that is sometimes manifested as skin eruptions which may be macular or resemble the rose spots of typhoid fever. Localization may occur, however, and meningitis and meningo-encephalitis are probably not so rare as has been supposed, while in some instances orchitis, cholecystitis, endocarditis, and other local manifestations have been reported. Pulmonary lesions with infiltration of the hilar glands or lung tissue proper are occasionally observed. Pulmonary infection has led many investigators to suspect infection by inhalation, and Elberg and Henderson[13] have shown that the guinea pig may be experimentally infected by an intake of about thirty-six microorganisms inhaled as an aerosol. Brucella infection may be associated with abortion and mastitis in the human female in rare instances. As indicated above, the entotoxin appears to contribute in large part to the pathogenesis of the disease, both directly[1] and indirectly as a hypersensitivity.[58]

Epidemiology.[24, 47] Brucellosis in man is probably always acquired from infected domestic animals; man-to-man transmission is a possibility but rarely, if ever, occurs. The commonest modes of infection in the United States are the use of raw milk from infected cattle, and direct contact with the flesh of infected animals, both cattle and swine. As indicated above, animals may be readily infected via the alimentary tract, and it is not unreasonable to suppose that man is infected in this way also. The discharge of *Br. abortus* in the milk of infected cattle, then, provides the opportunity for infection when the milk is ingested in the raw state, and in many instances undulant fever is acquired in this way. *Br. abortus* has been found in certified milk in a number of localities. The pasteurization of milk, of course, provides adequate protection from this source of infection.

The penetration of the unbroken skin by Brucella has been pointed out earlier. Man may be infected by the handling of the tissues of diseased animals or by close contact with other infectious material; presumably the bacilli enter through minute abrasions in the skin or possibly through the intact skin. Employees of slaughterhouses, veterinarians, sausage-makers, and butchers are, of course, particularly exposed to infection by this means, and the incidence of brucellosis in this group is disproportionately high with a tendency to be an occupational disease. It is probable that most infections with *Br. suis* are acquired in this manner, although in some instances cattle are infected with this species and man acquires a *suis* infection via raw cow's milk. The epidemiology differs somewhat from one locality to another. In Iowa incidence is highest among packing house workers and male farm workers, and in Indiana direct contact appears to be most important. In Utah, however, infected dairy products are the most important source of infection. In general, brucellosis is more common in males than females, in a ratio of about 4:1.

Laboratory infections with Brucella are very common, and even the most skilled workers have acquired undulant fever through working with these bacteria. Meyer and Eddie[36] reviewed seventy-four cases up to 1940 of which forty-four occurred in competent bacteriologists. These infections are, in all probability, a consequence of handling infectious material and penetration of the skin by the microorganisms.

Undulant fever may also be acquired

by drinking raw goat's milk, but infections with *Br. melitensis* from these animals are thought to be relatively infrequent in the United States. A number of cases have been found in North Carolina, Kansas, and Texas, and it is known to occur in the Southwest in general where goat's milk is consumed. In Arizona the incidence of *Br. melitensis* infection is directly related to the goat population and has declined coincident with a reduction in the numbers of goats, while *Br. abortus* infection has remained high.

It has been shown experimentally that brucellosis may be transmitted by mosquitoes and biting flies, but at present there is no indication that this mode of transmission is of any significance in nature. Water is apparently not a vehicle of transmission; the single water-borne outbreak that has been reported was in the nature of a laboratory accident.[39]

The prevalence of human brucellosis is not known with any degree of precision. The reported incidence of brucellosis reached a peak in 1947 with 6321 cases, declined to 3139 in 1951, 1300 in 1956, and to 636 in 1961; the numbers of reported cases tend to reflect general interest, or laxity in reporting. The midwestern states, Iowa, Minnesota, and Wisconsin, contributed 25 per cent of all cases in the 10-year period 1942 to 1951 and 50 per cent of cases reported in 1951. The disease is found in rural areas for the most part, and the incidence in urban areas is very low. The incidence is high in the midwestern states and, in geographical distribution, tends to parallel the extent of the hog-raising industry. The incidence of brucellosis in cattle is relatively high, about 4 per cent of adult females, and more or less uniform throughout the country. Swine brucellosis is not so common; about 3 per cent of hogs show significant agglutinin titers. The estimated annual loss to the livestock industry is about 100 million dollars.

Bacteriological Diagnosis.[27, 52, 61] The laboratory diagnosis of brucellosis involves the demonstration of the causative microorganism and of specific antibodies. When the bacterium can be isolated and identified the diagnosis is solidly established, but the presence of antibodies indicates only an immune response to what may have been a past rather than present infection and is, therefore, only suggestive.

Brucella is more often found in the blood stream in man, particularly in the pyrexial period, but cannot always be isolated. Blood or blood clot is added to tryptose broth in 2 to 5 ml. amounts and incubated in an atmosphere containing 25 per cent CO_2. The enrichment culture should be subcultured at 4-day intervals and, if subcultures are negative, carried for a period of not less than 3 weeks. For subculture agar plates of liver infusion or tryptose agar should be inoculated. The bacteria may be identified by agglutination with antiserum; differentiation between *Br. melitensis* and *Br. abortus-Br. suis* may be made by agglutination with type-specific antiserum, and the culture tested for H_2S production and growth in the presence of thionine and basic fuchsin.

A presumptive diagnosis made on clinical grounds may be partially, but by no means absolutely, confirmed by an agglutinin titer of 1:320 or higher or by a rising antibody titer during the course of the disease. Unfortunately, agglutinin titers differ from one laboratory to another, and there is pressing need for the use of a standard agglutinating antigen and agglutination procedure.[15, 55, 62] A rapid test has been devised by Ruiz Castaneda[50] which does not require laboratory facilities.

Blocking antibody occurs which interferes in the direct agglutinin titration.[56] There has been considerable interest in the diagnostic utility of incomplete, or blocking antibody titration by the antiglobulin method.[10, 16, 17, 22, 64, 68, 71, 74] The significance of such titers is somewhat uncertain; it is believed that incomplete antibody tends to be formed relatively late in the disease, and possibly may be taken to indicate the presence of active infection, *e.g.*, continued antigenic stimulus.

Interpretation of the agglutinin re-

sponse is also complicated in some instances in which the individual has received cholera vaccine, for Brucella and the cholera vibrio contain common antigens. The allergic response to Brucella cell substance and demonstrable as a skin reaction of the delayed type is now regarded as quite unreliable for diagnostic purposes.

The agglutination test is widely used in the diagnosis of brucellosis in cattle. Agglutinins are present in milk as well as serum, and in the latter case a "ring test" (the Abortus Bang Ringprobe, or ABR test) is commonly used. It consists of mixing 1 drop of hematoxylin-stained bacteria with 1 ml. of milk, incubating for 1 hour at 37° C., and reading; the agglutinated bacteria are carried to the surface with fat globules to form a colored ring.

Chemotherapy.[14, 51, 59] Chemotherapy of brucellosis has been disappointing, and the disease responds only irregularly and temporarily to administration of sulfonamides and the antibiotics. There is some evidence that combined therapy, as with sulfadiazine plus antibiotics or a combination of tetracyclines and streptomycin, may terminate acute clinical episodes, but relapses occur, ranging from 20 to 50 per cent. Of the antibiotics, the tetracyclines are considered to be the most effective.

Immunity.[28] The resistance of calves and nonpregnant cows to clinically apparent brucellosis is clearly an expression of natural immunity, though the older animals respond to the microorganism with the production of antibodies and the development of an increased resistance to subsequent infection. Man likewise appears to have a high degree of natural resistance to the infection, and it is probable that there are many more infections than clinical cases of brucellosis. In the series studied by Huddleson and Munger in which exposure to infection was known, only about half the individuals showing evidence of infection by an immune response had clinically apparent disease.

In man the immune response is evidenced by the appearance of agglutinins, opsonins, bactericidal antibody,[5] and hypersensitivity to preparations (*brucellergen*) of the cell substance of the bacteria. It is not clear, however, that this response is associated with an increased resistance, *i.e.*, effective immunity, to the infection. The available evidence suggests that an effective immunity is primarily a cellular immunity.[26] Consistent with this, the therapeutic use of antiserums in human brucellosis has given disappointing results.

Vaccines prepared as suspensions of killed bacteria, while stimulating the formation of antibody, have not resulted in an effective immunity, but the use of living, avirulent bacilli has given encouraging results. A smooth, avirulent strain known as strain no. 19 has been the most widely used for vaccination. It differs from virulent strains of *Br. abortus* in that it does not require carbon dioxide, is relatively avirulent for the guinea pig, and is specifically inhibited by 0.4 mg. per liter of thionine blue, while other strains are not. This strain is not completely avirulent for man, and several accidental infections have occurred in veterinarians.[53, 63]

The use of this vaccine in calves has given some measure of control of bovine brucellosis. It does not immunize against the infection, but sharply reduces abortion and the shedding of virulent bacteria by infected animals. It has not been used for human immunization in this country, but has been tested extensively by Russian workers[43, 73] who have reported that the incidence of the disease is reduced to as much as tenfold by vaccination; and when the disease does occur in immunized persons its severity is reduced and relapses are much less frequent.

BRUCELLA BRONCHISEPTICA

This microorganism is very similar to *Br. abortus* but is motile and highly aerobic. It does not produce hydrogen sulfide. It is immunologically related to *Br. melitensis* and *Br. abortus* but can be sep-

arated from them by agglutinin-absorption tests. It also resembles *Hemophilus pertussis* both culturally and immunologically. Originally isolated from dogs ill with distemper, it is not now generally believed to stand in any causal relation to that disease. It is, however, frequently found as the cause of bronchopneumonia in guinea pigs and other rodents.

REFERENCES

1. Abernathy, R. S., and Spink, W. W.: Studies with Brucella endotoxin in humans: The significance of susceptibility to endotoxin in the pathogenesis of brucellosis. J. Clin. Invest., 1958, **37:**219–231.
2. Altenbern, R. A., Ginoza, H. S., and Williams, D. R.: Metabolism and population changes in *Brucella abortus*. I. Roles of alanine and pantothenate in population changes. J. Bact., 1957, **73:**691–696.
3. Altenbern, R. A., Williams, D. R., Kelsh, J. M., and Mauzy, W. L.: Metabolism and population changes in *Brucella abortus*. II. Terminal oxidation and oxygen tension in population changes. J. Bact., 1957, **73:**697–702.
4. Biberstein, E. L., and Cameron, H. S.: The family Brucellaceae in veterinary research. Ann. Rev. Microbiol., 1961, **15:**93–118.
5. Bienvenu, R. J., Jr., Rode, L. J., and Schuhardt, V. T.: Microcolony brucellacidal test. J. Bact., 1961, **81:**684–687.
6. Braun, W., et al.: The effects of metabolites upon interactions between variants in mixed *Brucella abortus* populations. J. Bact., 1951, **62:** 45–52.
7. Braun, W., Pomales-Lebron, A., and Stinebring, W. R.: Interactions between mononuclear phagocytes and *Brucella abortus* strains of different virulence. Proc. Soc. Exptl. Biol. Med., 1958, **97:**393–397.
8. Cluff, L. E., Trever, R. W., Imboden, J. B., and Canter, A.: Brucellosis. II. Medical aspects of delayed convalescence. Arch. Intern. Med., 1959, **103:**398–405.
9. Cruickshank, J. C.: Sodium diethyldithiocarbamate and oxime in the differentiation of Brucella species. J. Hyg., 1955, **53:**305–312.
10. Cruickshank, J. C.: Incomplete antibodies in experimental Brucella infection. J. Hyg., 1956, **54:**562–568.
11. Dishon, T., and Olitski, L.: Lethal Brucella infections in white mice produced with the aid of the mucin technic. Proc. Soc. Exptl. Biol. Med., 1949, **71:**698–700.
12. van Drimmelen, G. C.: "Species" of Brucella characterized by phage lysis. Bull. W. H. O., 1960, **23:**127–130.
13. Elberg, S. S., and Henderson, D. W.: Respiratory pathogenicity of Brucella. J. Infect. Dis., 1948, **82:**302–306.
14. Farid, Z., Miale, A., Jr., Omar, M. S., and van Pennen, P. F. D.: Antibiotic treatment of acute brucellosis caused by *Brucella melitensis*. J. Trop. Med. Hyg., 1961, **64:**157–163.
15. Feinberg, R. J., and Wright, G. G.: Factors influencing the agglutination titration in human brucellosis. J. Immunol., 1951, **67:**115–122.
16. Ferris, A. A., Stevenson, W. J., and Lewis, F. A.: The anti-globulin sensitization test as applied to Brucella infections: A preliminary report. Med. J. Australia, 1953, **1:**619–621.
17. Fey, H., and Bürki, F.: Erfahrungen mit der serologischen Diagnose der menschlichen Brucellose durch den Blocking- und Coombs-Test. Schweiz. med. Woch., 1953, **83:**573–575.
18. Freeman, B. A., and Vana, L. R.: Host-parasite relationships in brucellosis. I. Infection of normal guinea pig macrophages in tissue culture. J. Infect. Dis., 1958, **102:**258–267.
19. Ganado, W., and Bannister, W.: Bacteraemia in human brucellosis. Brit. Med. J., 1960, **i:**601–603.
20. Gay, K., and Damon, S. R.: Use of the chick embryo for isolation of Brucella. Multiplication of the organism in the yolk sac and selection of the embryo age optimal for isolation from blood. Pub. Health Repts., 1950, **65:** 1187–1194.
21. Gerhardt, P.: The nutrition of Brucellae. Bact. Rev., 1958, **22:**81–98.
22. Hall, W. H., and Manion, R. E.: Comparison of the Coombs test with other methods for Brucella agglutinins in human serum. J. Clin. Invest., 1953, **32:**96–106.
23. Harris, H. J.: Brucellosis (Undulant Fever). Clinical and Subclinical. Hoeber, New York. 2nd ed., 1950.
24. Hendricks, S. L.: Epidemiology of human brucellosis in Iowa. Amer. J. Pub. Health, 1955, **45:**1282–1288.
25. Holland, J. J., and Pickett, M. J.: Intracellular behavior of Brucella variants in chick embryo cells in tissue culture. Proc. Soc. Exptl. Biol. Med., 1956, **93:**476–479.
26. Holland, J. J., and Pickett, M. J.: A cellular basis of immunity in experimental Brucella infection. J. Exptl. Med., 1958, **108:**343–360.
27. Hosty, T. S., and Johnson, M. B.: Routine methods for cultivation and isolation of Brucella in a public health laboratory. Amer. J. Pub. Health, 1953, **43:**30–35.
28. Huddleson, I. F.: Immunity in brucellosis. Bact. Rev., 1942, **6:**111–142.
29. Huddleson, I. F.: Brucellosis in Man and Animals. Commonwealth Fund, New York. Revised ed., 1943.
30. Hutchings, L. M.: Swine brucellosis. Mich. State Coll. Vet., 1944, **4:**68, 69, 86.
31. Jones, L. M.: Comparison of phage typing with standard methods of species differentiation in Brucellae. Bull. W. H. O., 1960, **23:**130–133.
32. Martin, W. J., Schirger, A., Kelly, P. J., and Beahrs, O. H.: Brucellosis. Proc. Staff Meetings Mayo Clinic, 1960, 23:717–727.
33. McCullough, N. B., and Beal, G. A.: The biological stability of the genus Brucella. Bull. W. H. O., 1958, **19:**725–737.
34. McVay, L. V., Guthrie, F., Michelson, I. D., and Sprunt, D. H.: Isolation of Brucella from apparently healthy individuals. Proc. Soc. Exptl. Biol. Med., 1948, **69:**607–608.
35. Meyer, K. F., and Eddie, B.: Laboratory infections due to Brucella. J. Infect. Dis., 1941, **68:**24–32.
36. Meyer, M. E., and Cameron, H. S.: Species metabolic patterns within the genus Brucella. Amer. J. Vet. Res., 1958, **19:**754–758.
37. Miles, A. A., and Pirie, N. W.: The properties of

antigenic preparations from *Brucella melitensis*. I. Chemical and physical properties of bacterial fractions. Brit. J. Exptl. Path., 1939, **20:**83–98.
38. Miles, A. A., and Pirie, N. W.: The properties of antigenic preparations from *Brucella melitensis*. V. Hydrolysis and acetylation of the amino-polyhydroxy compound derived from the antigen. Biochem. J., 1939, **33:**1716–1726.
39. Newitt, A. W., Koppa, T. M., and Gudakunst, D. W.: Waterborne outbreak of *Brucella melitensis* infection. Amer. J. Pub. Health, 1939, **29:**739–743.
40. Pennell, R. B., and Huddleson, I. F.: Chemical constitution and biological properties of the endo-antigen of the Brucella group of microorganisms. Mich. State Coll. Agr. Expt. Sta. Tech. Bull. 156. 1937.
41. Pickett, M. J., and Nelson, E. L.: Speciation within the genus Brucella. IV. Fermentation of carbohydrates. J. Bact., 1955, **69:**333–336.
42. Pickett, M. J., Nelson, E. L., and Liberman, J. D.: Speciation within the genus Brucella. II. Evaluation of differential dye, biochemical and serological tests. J. Bact., 1953, **66:**210–219.
43. Poberezkin, M. N.: Features of brucellosis in vaccinated persons. J. Microbiol. Epidemiol. Immunobiol., 1960, **31:**305–310. (Translated from the Russian.)
44. Redfearn, M. S., and Berman, D. T.: Application of the gel-diffusion technique for typing brucellae. Bull. W. H. O., 1960, **23:**133–134.
45. Renoux, G.: Considérations sur la notion d'espèce dans le genre Brucella. Bull. W. H. O., 1958, **19:**739–745.
46. Renoux, G., Amarasinghe, A., and Sacquet, E.: Sur la classification des Brucella. Arch. Inst. Pasteur de Tunis, 1955, **32:**375–406.
47. Report: Joint FAO/WHO Expert Committee on Brucellosis. Second Report. World Health Organization Technical Report Series No. 67. Palais des Nations, Geneva, 1953.
48. Roux, J.: Virulence et toxicité des Brucella. Rev. d'Immunol., 1961, **25:**32–45.
49. Ruiz Castaneda, M.: Brucellosis. Ann. Rev. Microbiol., 1950, **4:**331–342.
50. Ruiz Castaneda, M.: The evaluation of rapid tests in human brucellosis. Bull. W. H. O., 1953, **9:**399–408.
51. Ruiz Castaneda, M.: Chemotherapy of brucellosis. Bull. W. H. O., 1957, **16:**443–446.
52. Ruiz Castaneda, M.: Laboratory diagnosis of brucellosis in man. Bull. W. H. O., 1961, **24:** 73–84.
53. Sadusk, J. F., Jr., Browne, A. S., and Born, J. L.: Brucellosis in man, resulting from *Brucella abortus* (strain 19) vaccine. J. Amer. Med. Assn., 1957, **164:**1325–1328.
54. Sanders, E., and Huddleson, I. F.: The influence of environmental conditions on the growth and dissociation of *Brucella abortus*. Amer. J. Vet. Res., 1956, **17:**324–330.
55. Schubert, J. H., and Herndon, J. F.: A study of certain factors affecting the agglutination test for brucellosis. Pub. Health Repts., 1950, **65:** 883–890.
56. Schuhardt, V. T., Woodflin, H. W., and Knolle, K. C.: Brucella-agglutinin-blocking factor in human sera. J. Bact., 1951, **61:**299–303.
57. Spink, W. W.: The Nature of Brucellosis. University of Minnesota Press. 1956.
58. Spink, W. W.: The significance of bacterial hypersensitivity in human brucellosis: Studies on infection due to strain 19 *Brucella abortus*. Ann. Intern. Med., 1957, **47:**861–874.
59. Spink, W. W.: Current status of therapy of brucellosis in human beings. J. Amer. Med. Assn., 1960, **172:**697–698.
60. Spink, W. W., and Anderson, D.: Experimental studies on the significance of endotoxin in the pathogenesis of brucellosis. J. Clin. Invest., 1954, **33:**540–548.
61. Spink, W. W., McCullough, N. B., Hutchings, L. M., and Mingle, C. K.: Diagnostic criteria for human brucellosis. Report No. 2 of the National Research Council, Committee on Public Health Aspects of Brucellosis. J. Amer. Med. Assn., 1952, **149:**805–808.
62. Spink, W. W., McCullough, N. B., Hutchings, L. M., and Mingle, C. K.: A standardized antigen and agglutination technic for human brucellosis. Report No. 3 of the National Research Council, Committee on Public Health Aspects of Brucellosis. Amer. J. Clin. Path., 1954, **24:**496–498.
63. Spink, W. W., and Thompson, H.: Human brucellosis caused by *Brucella abortus* strain 19. J. Amer. Med. Assn., 1953, **153:**1162–1165.
64. Stevenson, W. J., Ferris, A. A., and Lewis, F. A.: A comparison between the cutaneous sensitivity test and a modified antiglobulin test in investigation of Brucella infection in abattoir workers. Amer. J. Hyg., 1954, **59:**133–139.
65. Stinebring, W. R., and Braun, W.: Brucellaphage. J. Bact., 1959, **78:**736–737.
66. Stone, W. S.: Brucellosis in horses and goats. J. Amer. Vet. Med. Assn., 1941, **99:**118–120.
67. Trever, R. W., Cluff, L. E., Peeler, R. N., and Bennett, I. L.: Brucellosis. I. Laboratory-acquired acute infection. Arch. Intern. Med., 1959, **103:**381–397.
68. Wagner, B. M., and Kuhns, D. M.: Coombs type of antibodies (antiglobulin) in brucellosis. Amer. J. Clin. Path., 1953, **23:**185–189.
69. White, P. G., and Wilson, J. B.: Differentiation of smooth and nonsmooth colonies of Brucellae. J. Bact., 1951, **61:**239–240.
70. Wilson, J. B., and Dasinger, B. L.: Biochemical properties of virulent and avirulent strains of Brucellae. Ann. N. Y. Acad. Sci., 1960, **88:** 1155–1166.
71. Wilson, M. M., and Merrifield, E. V. O.: The antiglobulin (Coombs) test in brucellosis. Lancet, 1951, **ii:**913–914.
72. Wundt, W.: Die Biochemie und Serologie der Brucellen. Ergebn. Mikrobiol. Immunitätsf., 1961, **34:**120–179.
73. Zdrodowski, P., Vershilova, P., and Kotlarova, H.: Immunological research on brucellosis and human immunization against this infection by means of an attenuated live vaccine. J. Infect. Dis., 1957, **101:**1–7.
74. Zinneman, H. H., Glenchur, H., and Hall, W. H.: The nature of blocking antibodies in human brucellosis. J. Immunol., 1959, **83:**206–212.

Chapter 25

PASTEURELLA AND ACTINOBACILLUS

Hemorrhagic Septicemia; Plague; Tularemia; Glanders; Actinobacillosis

The group of bacteria making up the genus Pasteurella consists of biochemically inactive, nonmotile, gram-negative bacilli which often show bipolar staining. The group is relatively homogeneous, with the exception of the tularemia bacillus which is set apart by its fastidious nutritive requirements and certain other characteristics, and differentiable by physiological properties as shown in the accompanying table. These bacteria are primarily pathogens of lower animals.[16] The etiological agent of fowl cholera was among the first of them to be described, and was the bacterium used by Pasteur in his early studies on immunity.

Pasteurella Multocida. (*Pasteurella septica.*) The group of animal diseases loosely designated as hemorrhagic septicemias, and characterized by large and small hemorrhagic areas found in the subcutaneous tissues, serous membranes, muscles, lymph glands, and throughout the internal organs, are considered to be caused by a single species, *Past. multocida* or *Past. septica.* Strains may differ widely in virulence and a certain degree of host specificity and for many years were given corresponding names, *viz.*, *Past. aviseptica* or *Past. avicida,* the cause of fowl cholera and pathogenic for mammals as well; *Past. muriseptica* or *Past. muricida,* pathogenic for rodents but not fowl; *Past. leptiseptica* or *Past. cuniculicida,* associated with contagious nasal catarrh, or "snuffles," of rabbits; *Past. suiseptica* or *Past. suilla,* the bacillus of swine plague which is pathogenic for both mammals and birds; and *Past. boviseptica* or *Past. bollingeri,* found in domestic animals.

These bacteria may be grown on the conventional nutrient agar mediums, and show no hemolysis on blood agar although the colonies may become darkened. They occur as distinct colonial types, smooth, mucoid and rough. The smooth forms are iridescent when examined by oblique transmitted light, and Elberg and Ho[54] described three types, the green or golden being the most virulent, and the red and blue of decreasing virulence; the noniridescent mucoid type is avirulent. They are separable into four groups on the basis of arabinose-xylose-dulcitol fermentations which approximate, but do not necessarily coincide with, immunological type; characterization as types is immunological rather than biochemical. Roberts differentiated four serotypes, designated I, II, III and IV, and this was carried fur-

Differentiation of Pasteurella Species

SPECIES	MOTILITY (GROWN AT 18–22° C)	GROWTH* ON MCCONKEY'S AGAR	INDOLE	H_2S	MALTOSE FERMENTATION**
Past. septica	−	−	+	+	−
Past. hemolytica	−	−	−	+	+
Past. pseudotuberculosis	+	+	−	+	+
Past. pestis	−	+	−	−	+

* sparse, disappearing after 2 to 3 days incubation
** acid, no gas

ther by Carter[28, 29] who designated the types B, A, C and D corresponding respectively to Roberts' types. The immunological specificity is attributable to specific polysaccharide, and is demonstrable by precipitin, capsular swelling and passive hemagglutination reactions; effective immunity is type-specific. There is an association between colonial morphology and antigenic characteristics[30]; specificity is a property of the smooth form, and mucoid strains are often not typable.

The pathogenicity of *Past. multocida* is sometimes considered to be open to question because of the relatively common occurrence of the bacteria in normal animals. It appears, however, that strains isolated at random are of widely varying virulence, and those found in normal animals are commonly the avirulent mucoid form.[27] Virulence appears to be associated with serotype to the extent that type B (I), which is largely confined to Southeast Asia, is the etiological agent of classic hemorrhagic septicemia occurring in cattle and other domestic animals. The other types are associated with fowl cholera and bovine and swine pneumonias in this country and in Europe. So far as is known these bacteria are essentially avirulent for man although occasional cases of human infection are reported.[18, 36, 96, 143, 146]

Pasteurella Hemolytica. This bacterium is closely similar to *Past. multocida*, but differentiable by its formation of indole and failure to ferment maltose, and is immunologically distinct. It appears to be the cause, or a common cause, of shipping fever of cattle,[31] but is apparently able to produce disease under natural conditions only when the resistance of the animals is reduced by stress. It is also a cause of ovine mastitis, and has been called *Past. mastitidis.*

PASTEURELLA PESTIS—THE PLAGUE BACILLUS[64, 72, 98, 109, 124, 125]

Plague is an ancient disease, originating in central Asia and/or central Africa. Whether the Biblical plague of the Philistines in 1320 B.C. was plague of Pasteurella etiology as contended by some, or an epidemic of dysentery and piles (the last is difficult to believe) as asserted by others, is uncertain, but the great pandemic during the reign of the Emperor Justinian in 542 A.D. was certainly plague.

Plague prevailed extensively through-Europe during the Middle Ages. It has been estimated that 25,000,000 persons, or one quarter of all inhabitants of Europe, perished in the "great mortality" or "black death" of the fourteenth century (1348–49). Few diseases have left so deep a mark on general literature. Boccaccio's *Decameron* contains one of the most vivid descriptions of the plague ever written, and Defoe's fictitious* *Journal of the Plague Year* provides a realistic picture of the devastation of London in 1665 by an outbreak of "black death" in which 70,000 persons perished.

* Defoe was only four years old in the year of the great plague.

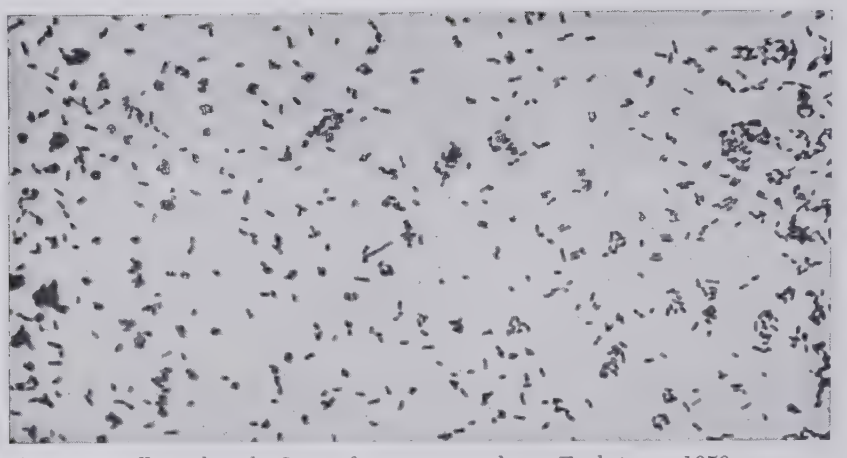


Figure 127. *Pasteurella multocida.* Smear from a pure culture. Fuchsin; × 1050.

For reasons that may be only partly conjectured plague has had irregular periods of quiescence and recrudescence. Western Europe has been practically free from the plague since the middle of the eighteenth century, and the disease began its first great extension in modern times with its appearance in 1893 in Hongkong and in 1896 in Bombay. Plague caused great loss of life in British India; official statistics show that in the period from 1896 to 1918 more than 10,000,000 deaths were due to this disease. In October, 1899, a case was recorded at Santos, Brazil; this is thought to be the first occurrence of the plague in the Western Hemisphere. Plague first appeared in the United States in San Francisco in 1900; it is assumed that it was introduced by infected rats from the Orient. The infection apparently spread to ground squirrels and other wild rodents in the western part of the country.

Plague continues its marked decline, with only 300 cases reported in 1959 in the entire world. This is considered to represent a continuation of its decline from its most recent epidemic prevalence, rather than a prelude to its extinction. In fact, the infection is now more firmly established in endemic foci than at any time in the past, and it may be a disease of the future as well as of the past.[6]

The plague bacillus, *Past. pestis,* was discovered almost simultaneously by Yersin and by Kitasato in 1894.

Morphology and Staining. The plague bacillus is a short, plump, ovoid rod 0.3 to 1.25 μ in length. In the body fluids the bacillis may occur in pairs, but long chains are rare, and, in general, there is no characteristic arrangement. The bacilli are nonmotile and a surface slime layer, polypeptide in nature, is present. Whether this substance, which is a significant element in virulence (see below), makes up an envelope, as it is usually called, or a capsule, is a matter of some discussion.[5, 39, 56] Involution forms are common, especially in older cultures, and coccus shapes, large rods, and gigantic swollen forms may be observed. The tendency of the plague bacillus to aberrant morphology is accentuated by cultivation on mediums containing 3 to 4 per cent sodium chloride; the appearance of involution forms in 24-hour cultures on salt-containing mediums has been regarded by some as a characteristic of differential value.

Colonies on nutrient agar or gelatin have a delicate, droplike appearance, with a round, granular center and a thin, granular, uneven margin. On blood, or other hemin-containing mediums, the colonies are dark brown in color, the pigmentation being derived from absorption of hemin from the substrate.[75]

The plague bacillus is uniformly gram-negative and shows a marked tendency

toward polar staining, *i.e.*, there are heavily stained areas at the ends of the cell separated by a lightly stained area in the center. For good bipolar staining the smear should be air-dried and fixed in alcohol. The usual aniline dyes, such as methylene blue, are satisfactory. The plague bacillus is best demonstrated in tissue sections by a polychrome stain.

Physiology. *Past. pestis* is not nutritionally fastidious, and growth occurs on all the ordinary culture mediums, although in peptone water it is very poor. Unselected strains require amino acids as sources of nitrogen, and certain amino acids appear to be essential for some strains,[71, 132] and apparently bacterial vitamins are not required. Casein hydrolysate has been widely used as a base for liquid mediums, for the production of vaccine at the Haffkine Institute for many years, and more recently in studies of toxin and other antigenic products,[66, 70, 140] but hematin, blood, or possibly reducing agents are required for growth on the surface of a solid medium.[67] Unlike the case with most of the bacteria pathogenic for man, a temperature of 25° to 30° C. is more favorable than one of 37° C., and the limiting temperatures for growth are —2° C. and 45° C. In any case, the colonies on solid mediums grow slowly and never attain a large size. The plague bacillus is aerobic and facultatively anaerobic.

Sugar fermentations are variable, including that of glycerol, and a small amount of acid but no gas is produced. Neither coagulated serum nor gelatin is liquefied, and indol is not produced. Nitrates may be reduced to nitrites, and a small amount of hydrogen sulfide is formed. On potato and in milk multiplication is slow and scanty; milk is rendered slightly acid but is not curdled.

Devignat has distinguished three physiological varieties of the plague bacillus.[45, 63, 123] Variety I, *Past. pestis orientalis,* does not ferment glycerol but reduces nitrate; variety II, *Past. pestis antiqua,* ferments glycerol and reduces nitrate; and variety III, *Past. pestis mediaevalis,* ferments glycerol and does not reduce nitrate. The primary foci of infection of variety I are in India, Burma, and south China; variety I was the agent of oriental plague which caused the 1894 epidemic and it is also responsible for sylvatic or wild rodent plague in the western United States. Variety II, probably the oldest, came from Transbaikalia, Mongolia, and Manchuria in central Asia, moved west with the Aryan invasions, and followed the valley of the Nile into central Africa, leaving foci persisting to the present time. It also moved back toward the Mediterranean in the sixth century and is believed to have been responsible for the Justinian plague that spread through the Roman Empire. It has since disappeared from Europe and has remained isolated in Africa. Va-

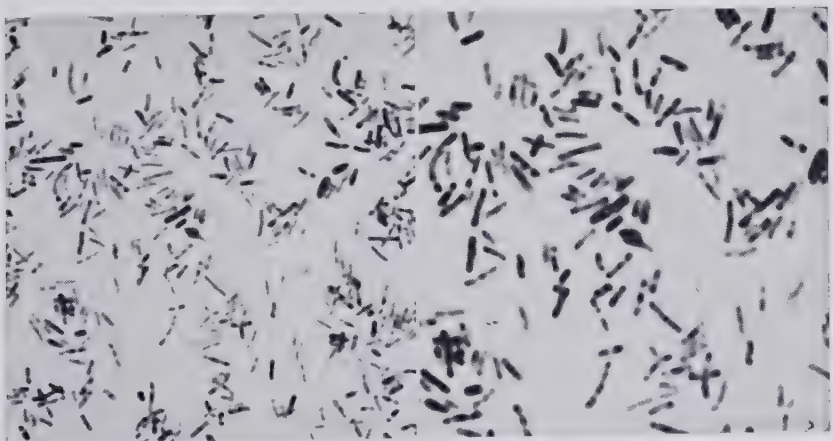


Figure 128. The plague bacillus. Smear from pure culture; fixed in methyl alcohol and stained with methylene blue to show bipolar staining. Note the involution forms present even at 24-hours' incubation. *Left,* × 1050; *right,* × 1800.

riety III, possibly arising by a slow transformation of variety II, spread from the Caspian Sea throughout the whole of Europe, causing the Black Death, and established itself in endemic foci.

The plague bacillus does not exhibit any marked resistance to deleterious influences. Exposure to drying, particularly at the higher summer temperatures, kills it within a short time. The bacillus is quite sensitive to the action of sunlight and chemical disinfectants; it is killed, for example, by 0.5 per cent phenol in 10 to 15 minutes and by heating to 55° C. in about the same time. Cultures kept in the refrigerator, however, remain viable over long periods. In general, *Past. pestis* does not survive long outside the animal body and disappears rapidly from soil, water, and buried cadavers.

Toxin. The toxicity of the plague bacillus appears to be intermediate between endotoxin and exotoxin. The toxicity is not associated with somatic glucolipid-polypeptide complexes as in the case of the enteric bacilli, but, while it appears to be protein in nature, it does not diffuse freely into the surrounding medium like the diphtheria, tetanus, and botulinus toxins. Toxic extracts have been prepared by freezing and thawing of suspensions, followed by centrifugation and filtration, and by autolysis and separation of the toxic supernatant. Toxicity in soluble form may also be prepared by phage or bile salt lysis of the bacilli.

On local inoculation the toxin produces edema, often followed by necrosis, and the general effect appears to be largely on the peripheral vascular system and liver to produce shock which is irreversible when a lethal dose is given. It appears to have no selective action on the heart or central nervous system. The pathology in toxin-sensitive animals—mice and rats—is closely similar to that produced in infection, but this similarity is not observed in other animals.[134]

This murine toxicity has been prepared in highly purified, antigenically homogeneous form by salting out procedures and electrophoretic fractionation of extracts of acetone-dried bacilli.[1, 2, 3] This material contains 18 amino acids, but no polysaccharide or phosphorus, has a molecular weight of about 74,000, and a mouse LD_{50} of 0.1 μg. or less.[14] It splits DPN, inhibiting DPN-dependent reactions such as the oxidation of keto acids[4] and inhibits the respiration of rat and mouse heart mitochondria, but not that of mitochondria from toxin-resistant animals such as the rabbit, dog, monkey and chimpanzee.[118] There is evidence[35, 87] which suggests that the murine toxin does not account for all the toxicity of the plague bacillus.

Virulence.[22, 55, 142] The murine toxin is found in both virulent and avirulent strains of the plague bacillus, and interest in matters such as variation and antigenic structure has centered largely around the question of virulence. The usual S-R dissociation occurs, but the colonial morphology, and associated properties, is not related to virulence since both S and R forms may be either virulent or avirulent.[51, 52, 53, 94] Similarly, the plague bacillus is antigenically homogeneous in that serotypes, in the conventional sense, do not occur, but the antigenic structure is complex as shown by sensitive techniques such as gel-diffusion.[15, 34, 40] The murine toxin is one antigenic component, and the polysaccharide-protein envelope substance, or antigen, designated Fraction 1, is another which is associated with virulence, but either or both may be present in virulent strains.

More recently the various factors associated with virulence have been clarified. The presence of Fraction 1 is associated with the resistance of the bacilli to phagocytosis, but doubt as to its indispensability to virulence is raised by the occurrence of virulent strains lacking, or containing only very small amounts of Fraction 1; such strains have been isolated in the laboratory as mutants[21] and in the naturally occurring fatal infection in man.[148] Two antigens, designated V and W and demonstrable by gel-diffusion, have been described,[23, 24, 25] which appear to be invariably associated with virulence; they do not occur separately,

The Known Virulence Determinants in *Pasteurella Pestis

PROPERTY					LD_{50}	
PURINE INDEPENDENCE**	PIGMENT ON HEMIN AGAR***	CAPSULAR ANTIGEN	VW ANTIGEN	CA^{++} DEPENDENCE	MOUSE	GUINEA PIG
+	+	+	+	+	<10	<10
−	+	+	+	+	$>10^8$	$>10^9$
+	−	+	+	+	$>10^8$	$>10^9$
+	+	−	+	+	<10	$\sim10^4$
+	+	+	−	−	$>10^8$	$>10^9$
+	+	+	+	−	$>10^8$	$>10^9$

* Prepared by Robert R. Brubaker.

** Strains lacking only the ability to synthesize purines are fully virulent when purine is inoculated simultaneously with the challenge dose.

*** Strains lacking only the ability to form pigment are fully virulent when Fe^{++} is inoculated simultaneously with the challenge dose.

but may vary in relative amount, and are thought to relate to the resistance of the bacilli to phagocytosis. Certain nutritional requirements have also been associated with virulence, or avirulence; one a temperature-dependent requirement for calcium[71] of virulent strains, and the others are nutritional deficiencies occurring in avirulent mutants. Of the latter, one relates to pigmentation of colonies on hemin-containing mediums noted earlier; nonpigmented mutants are avirulent unless the animal is inoculated concurrently with hemin or ferrous iron.[75, 76, 77] The other is a purine deficiency mutant for which virulence is similarly demonstrable when the deficiency is supplied at the time of inoculation.[20] Such restoration of virulence to deficient mutants is presumably a matter of supplying nutritive requirements not satisfied by the tissues of the host, *i.e.*, an effect on bacterial growth *in vivo*. Maintenance of virulence in culture is markedly influenced by environmental factors.[44, 60, 117, 122] In summary, a virulent strain of *Past. pestis* may be defined as a toxigenic, encapsulated strain containing the VW antigen complex, forming pigmented colonies on hemin-containing mediums, and able to survive and grow in macrophages.[32]

Pathogenicity for Man. Plague in man appears most commonly in two forms, the bubonic or glandular plague and plague pneumonia. In the bubonic type the symptom complex is characteristic, and diagnosis on clinical grounds is relatively simple. From the buboes, which may be either primary or secondary, bacilli may pass over into the blood; in fatal cases the bacteria often multiply in the blood extensively. The case fatality is 60 to 90 per cent. Primary plague septicemia can also probably occur. There are sometimes subcutaneous hemorrhages. During the plague epidemics in the Middle Ages such hemorrhages seem to have been more frequent than at present, and the dark spots to which they give rise were the origin of the popular name, "Black Death."

Plague pneumonia[110] occurs secondary to the glandular infection and may be transmitted to give rise to primary plague pneumonia. Pneumonic plague is usually fatal. In this variety the sputum may contain enormous numbers of plague bacilli, and the infection is spread from man to man by droplets. Because of this direct spread pneumonic plague is by far the more dangerous type. The extensive outbreak of pneumonic plague in Manchuria in 1910 to 1912 is said to have caused approximately 60,000 deaths, the case fatality being practically 100 per cent. Both bubonic and pneumonic plague have been produced experimentally, giving in the monkey diseases which closely resemble the human infection.[48, 73, 129, 139, 141]

A primary infection of the skin with the plague bacillus sometimes occurs (cutaneous plague) but does not seem to be common. Cases of mild plague, the so-called pestis minor, are met with in some

epidemics, but it is probable that healthy persons do not carry plague bacilli for long, if at all. The occurrence of intestinal plague in man has never been clearly established.

Epidemiology. Plague is a disease of rodents which also occurs in man as sporadic cases and in epidemic form. The susceptible rodents fall into two groups: the domestic rodents, or rats; and various wild rodents. Of the former, the black house and ship rat, *Rattus rattus,* and the less susceptible grey sewer rat, *R. norvegicus,* are most commonly involved, and the Egyptian rat, *R. alexandrinus,* may be infected also. Although both the feces and urine of infected rats may contain plague bacilli, and rats may be infected by feeding upon rats dead of the disease; the infection is most often transmitted by fleas. The Indian rat flea, *Xenopsylla cheopsis,* is a common, and perhaps the most effective, vector, but the rat flea of North America and Europe, *Ceratophyllus fasciatus,* may also transmit the disease. Plague bacilli are present in the blood during the acute disease, as many as 100 million per ml., and the flea is infected by feeding on the diseased rat.

When the plague bacilli are taken into the stomach of the flea they multiply, but do not spread to other parts of the body, and on microscopic examination large masses of bacilli may be seen in the infected flea. In some the bacilli multiply so rapidly that the bacterial mass mechanically obstructs the proventriculus so that little or no food may pass. In the effort to feed, plague bacilli are mixed with the drawn blood of the host, and regurgitated into the bite. The bacilli are also discharged in the feces, and infection may occur by contamination of the bite wound with fecal pellets.

Figure 129. The plague bacillus in the blood of an infected mouse. × 2000. (Douglas and Wheeler.)

A wide variety of wild rodents can be, and are, infected with plague, and this rodent disease, improperly referred to as sylvatic plague, constitutes the reservoir of infection. In the United States wild rodent plague was first observed in California in 1908, and has spread slowly eastward, reaching into Texas, Arizona, New Mexico, Oklahoma and Kansas. Rodents such as prairie dogs, ground squirrels (Citellus), wood rats (Neotoma) and mice, both Microtus and Peromyscus, may be infected,[57, 82, 83, 84, 97] and there is evidence that rabbits may be involved in some areas.[81] Various rodents are infected in other parts of the world,[131] the sisel, *Citellus pygmaeus,* in the northwest Caspian area, various species of Meriones in Kurdistan, the Indian gerbil, *Tatera indica,* in India, the field rat, *R. exulans,* in Java, etc.

It is well known that plague exists in two kinds of endemic centers, temporary and permanent, and the status of the domestic rat as a reservoir of infection has been doubtful. Similarly, a postulated role of the rat flea in the dissemination of human epidemic plague has been subject to serious exception with the occurrence of epidemic plague in the absence of rats and their fleas. More recently the epidemiology of the disease has been considerably clarified. Extensive studies, beginning in Kurdistan, have made it clear (a) that the permanent reservoirs of infection are in relatively resistant wild rodents; (b) that foci of wild rodent plague tend to die out when the animals are susceptible to the disease; (c) that the domestic rat is no more than the "liaison rodent" between the wild rodent and man; and (d) that while infection is initially carried to man by ectoparasites of rodents, epidemic bubonic plague in man

is transmitted largely from man to man by human ectoparasites, notably the human flea, *Pulex irritans*.

The epidemiology of plague falls into a pattern on the basis of the foregoing. For example, in Kurdistan four species of wild rodents constitute the reservoir of infection: *Meriones libycus* and *M. persicus* are highly resistant to plague, and *M. tristrami* and *M. vinogradovi* are extremely susceptible. The persistence of plague is a consequence of the balance among these species, and the sedentary habits of the resistant species whose burrows act as reservoirs of infected fleas. On occasion a human infection is acquired from this source, and this gives rise to human plague in villages, which is transmitted by the human flea.[8] When the villages are small and scattered, and communications slow, the epidemic is limited and dies out. Throughout this permanent epidemic area, including southeastern Russia, the infection persists in very many small permanent foci of infection where the various Meriones species are closely intermingled. In Java the field rat is resistant, and plague persists in permanent foci; the domestic rat is relatively rare in rural areas, too restricted in range to spread the infection, and too susceptible to maintain it for any length of time.[10] Consequently, plague in Java is typically sporadic, brief, and limited to villages. In the United States Microtus and Peromyscus are resistant to plague and for that reason are probably a more important reservoir of infection than the more susceptible ground squirrel.

In contrast, the focus of infection in Mesopotamia remains temporary, occurring in susceptible rather than resistant wild rodents, and tends to die out unless periodically reactivated by importation of infection.[11] Similarly, in India the gerbil is somewhat resistant to plague infection, but not sufficiently so as to allow the development of permanent pockets of the disease, and plague in India appears to be epidemiologically unstable and will presumably die out unless infection continues to be re-introduced.[9] Introduction of infection into a healthy area can occur only where *R. rattus* is present, the epizootic is brief unless the more resistant *R. novegicus* is present, and these domestic

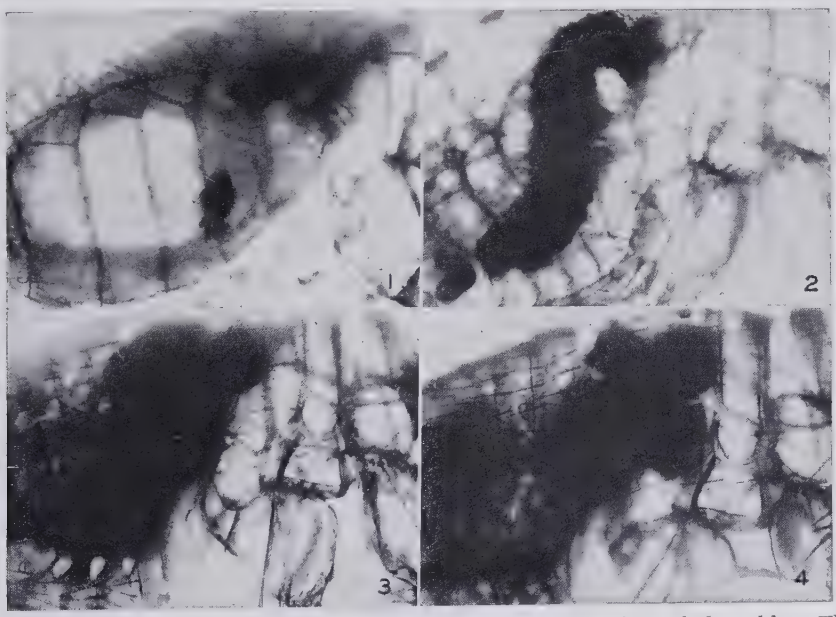


Figure 130. Plague bacilli in the infected flea; sections stained with methylene blue. The bacilli appear as dark stained masses. *1*, Flea on the ninth day after infection. *2*, Eighteenth day after infection; note the stomach and proventriculus packed with bacilli. *3*, Twenty-second day after infection; note the large mass of bacilli and the swollen proventriculus. *4*, Twenty-third day after infection; the proventriculus is further enlarged. (Douglas and Wheeler.)

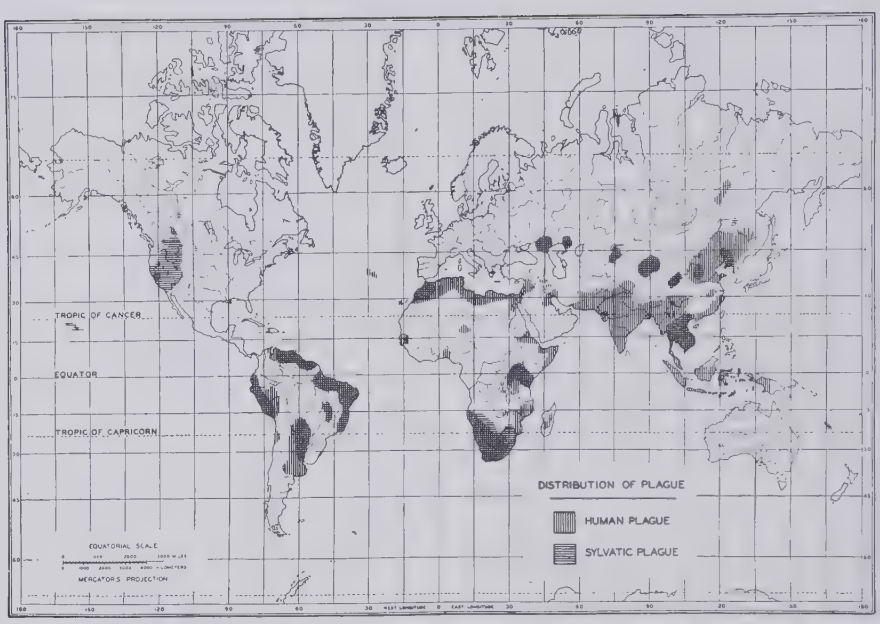


Figure 131. The world-wide distribution of human and sylvatic plague. Redrawn from map prepared by Army Medical Intelligence. (Based on Goode *Base Map* No. 201M. By permission of the University of Chicago Press.)

rats serve to infect the wild rodent population.

The prominent role of the human body louse in epidemic plague in man points up the significance of disinfestation as a control measure, together with chemoprophylaxis, in epidemics. In the longer view, control of the rat population will prevent introduction of new infection, and reduce the probability of human infections initiating epidemic spread. Control of wild rodent plague is a difficult matter, but encouraging results have been reported from Russia.[58]

Bacteriological Diagnosis.[7, 61] In man the bacilli are found in material aspirated from buboes, in cultures or smears of internal organs, especially spleen, and, in pneumonic plague, in the sputum. The presence of gram-negative, bipolar staining, ovoid bacilli is highly suggestive. Blood cultures, taken late in the disease, should be cultured first in broth. Other material may be inoculated directly on blood agar and glycerol agar. Cultures may be identified by cultural and biochemical characteristics and by agglutination in plague antiserum. The bacilli show some tendency to spontaneous agglutination, and the slide agglutination test is unsatisfactory. Postmortem diagnosis may be made by tapping the liver, lungs, and buboes, if present, with a syringe. The serous fluid is used for preparing smears and, after dilution in saline, for the inoculation of guinea pigs. Microscopic examinotion of smears often gives almost unequivocal diagnosis, but animal inoculation should always be carried out.

Guinea pigs may be inoculated subcutaneously or, with specimens that have undergone gross contamination and decomposition, by rubbing the material on the freshly shaved abdomen; the plague bacilli penetrate the minute abrasions while the contaminants do not. The animals die in 2 to 5 days; postmortem findings are characteristic and include subcutaneous and general congestion, congested spleen, granular liver, and pleural effusion. The bacilli may be found in spleen smears and elsewhere and cultured. It is important that the animal be freed of ectoparasites before inoculation. Plague in rodents may be diagnosed by postmortem findings, which are similar to those in the guinea pig, by microscopic and cultural demonstration of the bacilli, and by guinea pig inoculation. Of the serological reactions, passive hemagglutina-

tion appears to be the most sensitive.[92, 114, 115]

Chemotherapy.[103, 106] Streptomycin and sulfadiazine appear to be the most effective chemotherapeutic agents in plague. Prophylactic inoculation with sulfadiazine or sulfamerazine is reasonably effective for the protection of contacts. For therapeutic purposes, streptomycin is given for the first 4 to 5 days and then replaced by sulfonamide. In severe septicemic and/or pneumonic plague, the streptomycin may be supplemented with oral chloramphenicol or tetracyclines and antiserum prepared in the rabbit.

Immunity.[107, 108] Recovery from plague confers a solid immunity to subsequent infection. In this immunity phagocytosis appears to be of primary importance, functioning through opsonization to counteract the antiphagocytic properties of the envelope or capsule of the bacillus. It is probable that antibody to the envelope antigen, Fraction 1, plays a significant part in an effective immunity, and that to the VW antigen complex may be significant also. Antitoxic immunity does not contribute materially to effective immunity, and antitoxic serums are not protective.

While virulent strains are considered to be preferable for the preparation of killed vaccines, vaccines prepared with avirulent strains may also give an immunity as do certain living vaccines of avirulent strains. Various kinds of soluble preparations[33, 85, 137] produce an immunity under experimental conditions as indicated by protection tests in monkeys,[47] mice[17] or guinea pigs, but the immunity may not be of a high order. Differences in the response of animal species makes for some difficulty in assaying immunogenic potency, *e.g.*, a preparation producing an immunity in mice may not immunize guinea pigs.

Immunization of man has been carried out with vaccines made up of suspensions of killed or attenuated bacilli or with whole culture antigen. The vaccine used by the United States Army during World War II consisted of a suspension of 2000 million formalin-killed virulent plague bacilli per milliliter, and was given in two doses, 0.5 ml. and 1.0 ml., 7 to 10 days apart. The Haffkine Institute vaccine is a whole culture antigen. The plague bacilli are grown in nonantigenic casein hydrolysate medium decolorized with charcoal and containing Tween 80 to facilitate diffuse growth. It is standardized by immunogenic potency rather than by the number of bacilli contained in it.

Inoculation with living attenuated bacilli produces a solid immunity in experimental animals. The use of such vaccines in man has been investigated in South Africa with encouraging results, and a similar vaccine has been used in Indonesia in more than ten million inoculations without untoward results. These vaccines have been of particular interest in Russia also.[91]

PASTEURELLA PSEUDOTUBERCULOSIS

Past. pseudotuberculosis (*B. pseudotuberculosis rodentium*) resembles the plague bacillus closely, but causes a disease of rodents, particularly guinea pigs. It is differentiated from the plague bacillus by its motility at 18–22° C. (by stab culture in soft agar), its production of urease and fermentation of rhamnose and glycerol.[46, 62] It has been reported to grow abundantly as large opaque colonies on desoxycholate-citrate agar while *Past. pestis* grows only scantily as reddish pinpoint colonies.[145] It is less virulent for the guinea pig than the plague bacillus, and produces a toxin which differs from that of *Past. pestis* antigenically and in that rabbits and guinea pigs as well as rats and mice, are susceptible to it.[135] A number of agglutinative serotypes have been described.[89] The two species of Pasteurella share some antigenic components[41, 43, 128, 144] including the VW, or virulence antigen, complex,[26] and show a degree of cross protection.

The natural mode of infection is probably by way of the alimentary tract. Subcutaneous inoculation of guinea pigs proves fatal in 2 to 3 weeks, with caseous swellings and nodules ("pseudotuber-

cles," which have unfortunately given this bacterium its name) in various organs. *Past. pseudotuberculosis* has been found, though rarely, in animals other than the guinea pig. Human infection is rare and has been considered to be almost uniformly fatal; but less serious disease, commonly of lymphoid tissue and sometimes simulating acute appendicitis when the adenitis is mesenteric, is observed from time to time.[42, 90, 136]

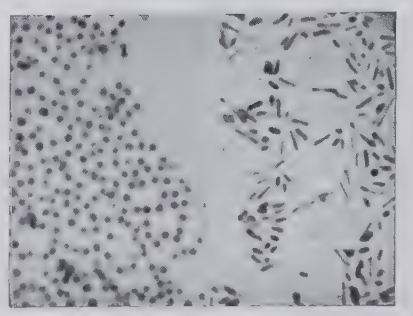


Figure 132. *Pasteurella tularensis.* Note change from coccoidal to bacillary form in 24 hours on fresh culture medium. (Francis.)

PASTEURELLA TULARENSIS[59]

Tularemia is a disease of rodents, rabbits in particular, that is transmitted to man either directly through the handling of the flesh of infected animals or indirectly through an insect vector. *Past. tularensis* (*Bacterium tularense*) was discovered by McCoy and Chapin in 1912 in a plague-like disease of the California ground squirrel. Tularemia in man, however, is contracted largely from the rabbit, and it was shown by Francis in 1919 that the disease known in Utah as deer fly fever is, in fact, tularemia transmitted from infected rabbits to man by the bite of the fly *Chrysops discalis.* Francis also found that *Past. tularensis* was present in rabbits sold in the markets of Washington, D. C., and that a disease known as rabbit fever was not infrequent among those in contact with the rabbits. In the United States 365 cases were reported in 1961. Human cases have been observed throughout the continental United States.

Morphology and Staining.[68] In culture *Past. tularensis* is a minute, gram-negative pleomorphic rod 0.2 μ in breadth and 0.3 to 0.7 μ in length; the coccoid form predominates in young cultures and the bacillary form in older cultures. In smears from the spleens of infected mice or guinea pigs the bacteria appear as coccoid forms in well-defined clusters. Capsules are present in the body; spores are not formed, and the microorganisms are nonmotile. The organism reproduces by a number of methods, including binary fission, budding, filament formation, and the like, and is regarded as closely related to

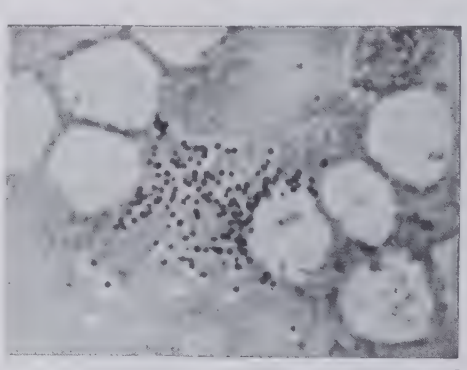


Figure 133. *Pasteurella tularensis;* coccoidal form; blood of rabbit. (Francis.)

the microorganisms of the pleuropneumonia group (Chap. 27). It is thought by some to be more closely related to Brucella than to Pasteurella and is, in fact, named *Brucella tularensis* by British workers; it has also been suggested that it be set apart and a new genus, Francisella, created for it.[121]

On solid mediums *Past. tularensis* forms minute, transparent, droplike colonies that are mucoid in consistency and readily emulsifiable. Colonial variants, including those associated with virulence, are more sharply characterized when observed by oblique transmitted light.[49, 50]

This bacterium is somewhat difficult to stain; methylene blue is not satisfactory, but either carbol-fuchsin or aniline gentian violet may be used. Bipolar staining may be observed.

Physiology. *Past. tularensis* differs sharply from the other members of this genus in that it will not grow on the ordinary mediums. It may be cultured on a coagulated egg-yolk medium or on blood dextrose cystine agar. Growth oc-

curs in casein or gelatin hydrolysate liquid medium supplemented with biotin, blood cell extract, and liver extract, and a medium of heart infusion, dextrose, cystine, and hemoglobin will support good growth. It has also been cultivated in chemically defined mediums[101, 149]; spermine is required, and the pH is critical and should not be higher than 7.5. It is aerobic and facultatively anaerobic, and its optimum temperature is 37° C. Glucose, maltose, and mannose are fermented, the fermentation of glycerol, levulose, and dextrin is irregular, and mannitol, galactose, xylose, trehalose, salicin, arabinose, adonite, sucrose, lactose, amygdalin, dulcitol, erythritol, inositol, inulin, raffinose, sorbitol, and rhamnose are not fermented. Differential fermentations are of no value. It is killed by exposure to 56° C. for 10 minutes. It has been reported that this bacillus contains an endotoxin.

Pathogenicity. Two clinical types of tularemia are recognized, one the glandular or ulceroglandular type, which is the more common, the other the so-called typhoidal type. In the first instance the acute stage of the disease is characterized by headache, pains, and fever, and a papule appears, frequently on a finger where presumably the bacilli enter the body, which later breaks down and forms an ulcer. The axillary and epitrochlear glands become painful and swollen and may break down with the discharge of purulent material. In persons infected via the conjuctiva, ulcers form on the inner surfaces of the eyelids, and the cervical and preauricular glands may become tender and somewhat swollen. In the typhoidal type of the disease there are no local symptoms.

During the first week of illness the bacilli may be present in the blood and have been cultured, although, in general, cultures made directly from man are not successful, and the bacillus is best isolated by guinea pig inoculation and culture from necrotic foci found in liver, spleen, and lungs of the pig on autopsy. Direct cultivation from the blood is rarely possible but has been accomplished, and it has been suggested that during the first week of the disease initial bacteremia may occur which, in fulminating cases, develops into septicemia. The bacilli can only rarely be found in smear preparations from human cases. In the experimental disease the bacilli are present in the lymph spaces and phagocytic cells of infected tissues, and their presence within the cells in enormous numbers has caused some workers to suggest an intracellular proliferation of the microorganisms similar to that of the rickettsiae. Agglutinins are present in the blood in the second week of the disease and may persist in diminishing amounts for at least as long as 18 years after recovery. The average duration of the disease is 2 to 4 weeks. The case fatality is low, 4.8 per cent, and the pathology of the disease in man is not well known.[102]

A variety of lower animals have been found to be naturally infected—in addition to the ground squirrels and rabbits noted above, wild rats and mice, woodchucks, opossums, beavers, coyotes, deer, red foxes, ground hogs, muskrats, hogs, skunks, dogs, cats, and lambs. In the western United States sheep infection, with a mortality as high as 10 per cent, serves as a source of human infection.[78] The infection also occurs naturally in some birds such as sage hens, grouse, and quail.

Of experimental animals, the mouse, guinea pig, and rabbit are susceptible in that order. The LD_{50} of a highly virulent strain is 10 or less for any of these animals, but as virulence declines it is evident first in the rabbit, then the guinea pig, and finally the mouse.[13]

Epidemiology. As indicated above, tularemia is acquired by man from lower animals either directly or indirectly. The bacilli may enter the unbroken skin of the guinea pig, and possibly this may occur in man through the dressing of infected rabbits and other animals, or the microorganisms may enter by means of minute abrasions on the skin of the hand. The bacteria may penetrate the intact skin; in the mouse the LD_{50} through the skin is two to five times that by mouth, and about 10^7 times the parenteral dose.[127] Eye infection occurs, and in fact, such infections were the first human infections with the bacillus observed. Over 90 per cent of the human cases in this country

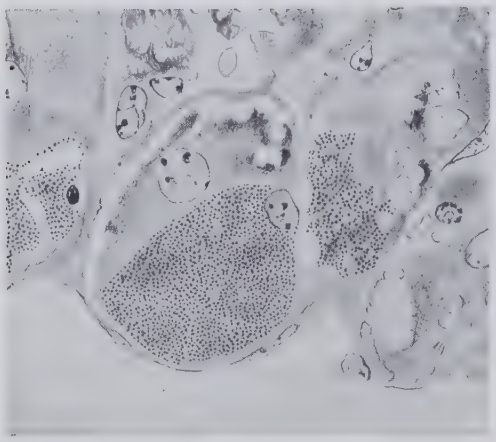


Figure 134. *Pasteurella tularensis* in hepatic cells of mouse. (Francis.)

are contracted from rabbits, and it is estimated that about 1 per cent of wild rabbits are infected. Jellison and Parker[79] have reported that the cottontail rabbit, species of Sylvilagus and S. *floridanus* in particular, is by far the most important source of infection in this country, accounting for more than 70 per cent of all human cases in North America. Many wild animals are naturally infected and Burroughs et al.[19] have compiled a list of forty-eight naturally infected vertebrates. Laboratory infection is not uncommon; fifty-six cases acquired from dissection were reported to 1940. *Past. tularensis* has also been found in streams and is perhaps associated with the epizootics occasionally observed in beavers. Present evidence indicates that fish cannot be infected with *Past. tularensis* and they probably play no part in the infection of water. Water-borne epidemics have occurred in Russia and Turkey. In this country infection of natural waters is relatively common in the northwestern states, and naturally occurring infections in beavers and muskrats are well known. In water and mud, infection may persist for many months, and available evidence suggests that the microorganisms may multiply there.[120] The infection is also transmitted by the ingestion of infected tissue[147] and this may be a factor too in maintenance of the reservoir of infection in carnivorous animals.

The transmission of tularemia by an insect vector is common. In addition to the deer fly, *C. discalis, Dermacentor andersoni, D. variabilis, D. occidentalis, Hemaphysalis leporis palustris, H. cinnabarina,* and *Ixodes ricinus californicus* may carry it. In all probability the wood ticks serve to disseminate the infection in the animal population, and it is of particular interest that the infection is transmitted from the adult tick to the egg, and both the larvae and the nymphs are infectious. Tularemia may, then, be in part maintained in the insect population. In Arkansas, for example, the disease is mainly tick-borne and is an occupational hazard among farm workers.

Tularemia has been found in all parts of the United States, in Japan where it is also known as yato-byo or O'Hara's disease, and in Central Europe, and extensive epidemics have occurred in Russia.

Bacteriological Diagnosis. As indicated above, *Past. tularensis* is difficult to cultivate from infected material. Specimens should be streaked on blood-dextrose-cystine agar. Characteristic minute, droplike colonies may appear in 3 to 5 days, but a culture should not be recorded as negative in less than 3 weeks. The procedure of choice is the intraperitoneal inoculation of a guinea pig with a saline emulsion of the specimen; relatively large amounts are required, usually 4 to 8 ml., of a fairly heavy emulsion. The animal should die in 5 to 10 days. The pathology is characteristic and includes hemorrhagic edema without pus at the site of inoculation, enlargement of the cervical, axillary, and inguinal lymphatics which contain dry caseous material, and small white necrotic areas in the liver and spleen. Smears and cultures may be made, but not infrequently the bacilli cannot be found or cultivated. In such instances the diagnosis depends on the guinea pig pathology.

Isolated cultures may be identified by specific agglutination. Conversely, patient's serum may be tested for agglutinins; a titer of 1:80 or higher is usually regarded as diagnostic if there are no Brucella agglutinins. In the event that Brucella is also agglutinated, the agglutination of *Past. tularensis* occurs more rapidly and to higher titer in tularemia.

Chemotherapy. The sulfonamides

are almost completely ineffective chemotherapeutic agents in tularemia. Of the antibiotics, streptomycin has been found highly effective, subject to the rapid acquiring of drug resistance by the microorganisms; the tetracyclines are effective, but chloramphenicol has only slight activity.

Immunity. An attack of tularemia confers an effective immunity, but it is not as solid as once thought. Two extremely well documented cases of reinfection among laboratory workers have been reported by Green and Eigelsbach.[65] Both had been immunized with vaccine; one subsequently acquired two clinical infections within 3 years, and the other, two infections within 6 years. Similar experiences have been reported by others.[105] The relation of antibody response to effective immunity is not clear; in any case agglutinin appears not to be closely related to protective antibody. Antibodies (agglutinins) to *Past. tularensis* show some cross-reaction with *Br. melitensis* and *Br. abortus.*

Prophylactic inoculation with vaccines is not successful in experimental animals in that it does not protect against the injection of virulent strains. There is some evidence, however, that vaccine prophylaxis in man confers a useful degree of protection.[80, 133]

ACTINOBACILLUS

The microorganisms grouped within the genus Actinobacillus are, like Mycobacterium (tubercle and related bacilli) and Corynebacterium (diphtheria and diphtheroid bacilli), more closely related to the fungi than many other bacteria. Nevertheless, they are classified with the Eubacteriales under the tribe Brucelleae, together with Brucella, Pasteurella, and Hemophilus. The distinctions between these last three are not sharp, and some of these bacteria are put in one genus by some workers, and in another by other workers. The microorganisms of the genus Actinobacillus tend to be set somewhat apart and include the causative agents of glanders and melioidosis, together with forms found in association with actinomycetes in certain kinds of actinomycosis (Chap. 32), producing disease which closely resembles certain of the fungous infections, known as actinobacillosis.

THE GLANDERS BACILLUS (ACTINOBACILLUS MALLEI)

Glanders is a disease seen, as a rule, only in the solipeds (horse, mule, ass) but is occasionally transmitted to other domestic animals, to wild animals, and to man. Early regarded by many as a spontaneous, noninfectious infection, the transmissibility of glanders was demonstrated in 1837 by Rayer, who infected a horse by inoculating it with material from a case of glanders in a human subject. The causative bacillus was discovered in 1882 by Löffler and Schütz, whose work was confirmed and extended by Kitt, Weichselbaum, and others.

Morphology and Staining. The glanders bacillus is a small rod, straight or slightly curved, usually with rounded ends, and often of irregular contour. Rather wide variations in size are observed; the average length may be taken as 2 to 5 μ and the average breadth 0.5 to 1 μ. In culture the bacilli tend to be shorter and more uniform in size than those observed in pus smears. In pus they are sometimes found within the leucocytes but more often occur free. There is no special arrangement in such smears, but in culture the bacilli may occur in pairs and, in older cultures, produce filaments with swollen ends in which true branching may be observed. The bacilli are nonmotile, nonencapsulated, and do not form spores.

Colonies on agar are small, round, con-

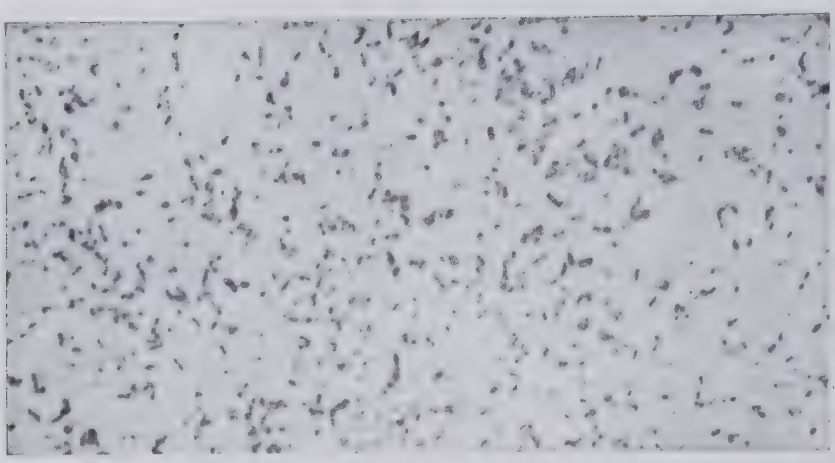


Figure 135. *Actinobacillus mallei* in pure culture. The generally poor staining is apparent, and bipolar staining may be observed in some of the cells. Methylene blue; × 1250.

vex and amorphous in consistency. They are translucent and yellowish in color and upon aging (8 to 10 days) become more opaque, and the center may become light brown. The growth on potato usually exhibits a characteristic appearance; clear, amber, honey-like colonies appear which may coalesce, and frequently the potato around the growth becomes tinged a greenish yellow, not unlike the discoloration produced by *Pseudomonas pyocyanea*. On horse blood agar the colonies are grayish green with browning of the medium but no hemolysis.

The glanders bacillus stains with the ordinary aqueous aniline dyes, though not readily. Best results are obtained with stains containing alkali, or a mordant such as phenol (Löffler's alkaline methylene blue, Ziehl's carbol-fuchsin). The bacilli are not acid-fast and are gram-negative. Cells from young cultures take the stain fairly uniformly, but those in older cultures stain irregularly, with a tendency to bipolar staining. Granules and coccus-like bodies within the cell take the stain somewhat more readily, and the bacilli have a beaded appearance in stained preparations. This irregular staining is due to the presence of lipid granules which do not stain with the usual dyes but may be demonstrated with Sudan black B or iodine-fuchsin.

Physiology. Growth occurs on ordinary nutrient mediums but is poor and slow on primary isolation. Forty-eight hours' incubation is generally necessary for the appearance on solid mediums of colonies 0.5 to 1 mm. in diameter. Growth is materially enhanced by the presence of glycerol, but glucose is without effect. A slightly acid reaction is favorable, and the optimum temperature is 37° C., though growth may occur over the range from 22° to 44° C. Growth on enriched mediums such as Löeffler's serum medium or horse-blood agar is not markedly better than on glycerol agar.[111]

The glanders bacillus is quite inactive biochemically. With the exception of glucose, there is no action on the usual carbohydrates, and even the glucose fermentation is irregular and variable from strain to strain. Coagulated serum is not digested, but gelatin may be liquefied under appropriate circumstances by some strains at least. At temperatures at which gelatin remains solid, growth is sparse and no liquefaction is observed, but if cultures are incubated at 37° C. for 26 to 40 days, the gelatin does not solidify on cooling. Indol is not produced, and nitrates are not reduced to nitrites; small amounts of hydrogen sulfide may be formed. Slight acid production sometimes occurs in milk, with coagulation by the tenth day of incubation and decolorization of the indicator in the lower part of the tube.

The bacillus is but slightly resistant to adverse physical and chemical agents, being readily killed by heat (55° C. for 10

minutes) and bactericidal chemicals. The most effective disinfectants are hypochlorites, iodine, and mercuric chloride. Desiccation experiments have not given uniform results; it is said that cultures dried on threads remain viable for 3 or 4 weeks. Pure cultures appear to be more resistant to desiccation than the bacilli in the nasal secretions from diseased animals, for infected discharges are usually sterile within a few days. Cultures die out in 4 to 6 weeks but may be maintained by transfer on glycerol agar.

Classification. The relation of the glanders bacillus to other bacteria is by no means clear. In certain respects, such as the occurrence of branching and the favorable effect of glycerol on growth, it resembles the Mycobacteria though it is not acid-fast. In other respects it is related to the Pasteurella species with which it is grouped as Actinobacillus.

Pathogenicity for Lower Animals. Under natural conditions the horse is chiefly affected, but cases are occasionally observed in the carnivora (cats, dogs, menagerie animals) and in goats and sheep. Swine and pigeons are slightly susceptible. Cattle and house rats are immune. Rabbits and guinea pigs are somewhat susceptible to experimental inoculation, and the hamster and ferret are the most susceptible of the usual experimental animals.[112]

The route by which the glanders bacillus usually enters the body of the horse has not been clearly determined. The mucous membrane of the nose, especially if slightly abraded, may become the portal of entry, as may the intact conjuctiva, which can be infected by contact with infectious material in 2 to 4 hours, sometimes in 30 minutes. Infection by inhalation must be rare, to judge from animal experiments. According to Nocard, infection occurs by way of the alimentary tract in the great majority of cases.

Glanders manifests itself in an acute and a chronic form, which run into one another, the latter frequently terminating in an acute attack. The acute form is ushered in usually by a chill and the appearance of a high temperature in advance of any local manifestation. In a few days the mucous membrane of the nose is inflamed and becomes studded with nodules, the lymphatic system becomes largely involved, and edematous swellings appear in various parts of the body. General symptoms become more grave, and death follows in from 8 to 30 days. The mule, and especially the ass, suffer commonly from the acute disease. The chronic form is the more usual type in the horse (90 per cent of cases). A great variety of symptoms and lesions have been noted in the latter animal, and the disease pursues most diverse courses in different individuals. The nasal membrane is often affected, and there is a profuse and infectious catarrhal discharge. Cutaneous glanders is known by veterinarians as *farcy*, the thickenings of the superficial lymphatics being termed "farcy buds" or "farcy pipes." In all forms of glanders there is a tendency to the production of nodules, which soften and pass over into ulcers. The glanders nodule has been considered by some writers to be structurally similar to the nodule formed by the tubercle bacillus (Chap. 30) but most observers agree that the former is a degenerative rather than a proliferative formation and that it is radically different from the tubercle.

Experimental inoculation with pure cultures has given positive results not only in the horse, in which the characteristic features of the disease are reproduced, but in guinea pigs, field mice, and other small rodents. The guinea pig is the commonly used animal for diagnostic purposes. Both in the natural and in the experimental infection the bacteria are found chiefly in the nasal secretions and in the contents of the young nodules; in the older ulcers they are relatively few in number. The blood, as a rule, contains glanders bacilli only in acute general infection.

Pathogenicity for Man. Veterinarians and others having to do with the care of horses are the most liable to contract glanders. Freshly isolated cultures are highly virulent, and a number of fatal infections have occurred among laboratory workers. The acute form of the disease is the more common in man, most cases

terminating fatally within 2 or 3 weeks, sometimes within a few days of their inception. As in the horse, the mucous membrane of the nostrils is often, although not invariably, affected. Occasionally the chronic form may appear and linger for months or even years, with spreading ulceration and other features closely resembling those observed in the horse. Recovery from chronic glanders may take place, or the disease may pass into the acute stage.[74, 104, 119]

In man the alimentary tract is certainly not the ordinary channel of entrance; meat from glandered animals has been ingested without resulting infection. Infection probably occurs through abraded skin.

Bacteriological Diagnosis. In prebacteriological days chronic glanders in the horse was frequently separated from other diseases only with difficulty and a considerable measure of uncertainty. At present the diagnosis of glanders is greatly facilitated by: (1) guinea pig inoculation; (2) the mallein test—(*a*) subcutaneous, (*b*) ophthalmic; (3) the agglutination method; (4) the complement-fixation test.

(1) ***Guinea pig inoculation.*** A male guinea pig is injected intraperitoneally with fragments of diseased tissue, scrapings from ulcers, or some of the nasal discharge from a suspected animal. A positive reaction is shown by the testicles becoming red and swollen, usually on the second or third day—the Straus reaction. Together with the orchitis (inflammation of the parenchyma of the testicle) there are severe general symptoms which usually culminate in 12 to 15 days. Grayish nodules are often found in the spleen and other internal organs. The test is not absolutely specific, for Kutscher and Nocard have shown that an analogous orchitis may be produced by other organisms besides the glanders bacillus. It is often, however, of value, especially when, for one reason or another, other tests are inapplicable.

(2) ***The mallein test.*** Mallein is the concentrated glycerol broth in which the glanders bacillus has grown; it is prepared in the same manner as tuberculin. The mallein reaction consists in a rise of temperature, accompanied by a pronounced local reaction, and in many cases, though not invariably, by more or less profound constitutional disturbances.

(*a*) Subcutaneous injection of mallein (the size of the dose varying according to the concentration) into a glandered horse is followed by the signs above noted, while in an animal not infected with glanders the temperature is slightly or not at all affected and the general symptoms are absent. The temperature of the suspected animal should be taken at 2-hour intervals before the injection is made, and after the injection at the ninth, twelfth, fifteenth, and eighteenth hours, at least. The increase of temperature in glandered horses varies from 1.5° to 2.5° C. above the normal and is distinctly high on the second day after injection. Healthy horses often show a distinct temperature increase on the first day after inoculation, but as a rule this disappears quickly. In the use of mallein, as in the tuberculin test, care must be taken to exclude other influences that disturb the normal temperature relations. Experienced observers lay much stress upon the appearance of swelling at the seat of inoculation. In a glandered animal the tumefaction is large, hot, and painful; it increases in size up to 29 to 36 hours, persists for about a week, and gradually disappears. In a healthy animal a swelling may occur, but it is never large and vanishes within 24 hours.

(*b*) The ophthalmic test consists in the introduction of the mallein, preferably in tablet form, into the conjunctival sac.

The conventional serological reactions, agglutination and complement-fixation, tend to give erratic results in that infected animals may show no evidence of antibody formation, and normal serum titers may be relatively high; normal horses, for example, may have agglutinin titers as high as 1:500. Serology is complicated by the fact that the antigenic structure of the bacteria is not established beyond early reports that there is some antigenic heterogeneity.

Chemotherapy. Glanders occurs so rarely that little is known concerning its chemotherapy. It has been reported that two chronic cases and six laboratory in-

fections were successfully treated with sulfadiazine.[38]

Immunity and Prophylaxis. Permanent immunity to glanders can neither be conferred by an attack of the disease nor produced by artificial means. Nocard fed with infectious matter three horses which had previously recovered from the disease and found that these animals showed no resistance superior to that of a healthy control animal. Similarly, Lobel, Schaaf, and Roza[99] were unable to produce an immunity in guinea pigs or horses by the inoculation of bile-attenuated avirulent bacilli. Chronic glanders may exist for years and is in no wise a warranty against the sudden development of an acute attack.

No very potent or characteristic toxic substance has been obtained from cultures of the glanders bacillus, and attempts at immunization with the products of this organism have been eminently unsuccessful. It is stated by a number of observers that repeated injections of mallein will exercise a curative action upon certain forms of recent infection, but experimentally mallein is without immunizing power. The serums of animals treated with mallein injections and the serums of naturally immune animals, such as cattle, are, according to most observers, totally devoid of any preventive or curative value. The most that has been accomplished in the way of immunization is a very moderate augmentation of resistance in dogs injected with small nonfatal doses of living cultures.

The experience of Great Britain shows that the disease may be practically eradicated by slaughtering every animal showing clinical signs of glanders, or giving a positive mallein test, and properly disposing of the carcass.

ACTINOBACILLUS WHITMORI (ACTINOBACILLUS PSEUDOMALLEI)

Melioidosis, a disease of rodents somewhat similar to glanders, is caused by *Actinobacillus whitmori*, a microorganism which closely resembles the glanders bacillus. It has been observed in Burma and also in Indo-China, where it is thought to be primarily a disease of the wild rat and is occasionally communicable to man,[88, 93] and three cases of apparently locally acquired infection have been reported in the Western Hemisphere.[12] *A. whitmori* differs from *A. mallei* in that it is motile, liquefies gelatin, and actively ferments carbohydrates. It grows considerably more rapidly, and its colonies on glycerol agar develop a wrinkled, corrugated surface and are quite different in appearance from those of *A. mallei*. A second colony type may be produced, however, which is very similar to the colonies of the glanders bacillus. *A. whitmori* is thought by some to be closely related to *Ps. pyocyanea* in many respects, and strains have been reported which produce pyocyanin and it has been called *Pseudomonas pseudomallei*. It has been reported that two thermolabile exotoxins are produced, one lethal and necrotizing, and the other lethal only.[37, 66] A heat-stable endotoxin, closely resembling those of the enteric bacilli and eliciting the Shwartzman reaction, has been described also.[130] An aggressin activity, apparently polypeptide in nature, has been found in sonic lysates of the bacilli; although not toxic itself, when given concurrently with the challenge inoculum it markedly increases mortality.[95, 100]

The characteristic lesion of the disease is a small caseous nodule which is found in man in almost any part of the body except the brain. The nodules may coalesce to form large areas of caseation in some cases and in others break down into abscesses. There is some reason to believe that melioidosis in man is often traumatic in origin, either the bacilli entering the injured tissues or the defense mechanism being broken down in part by the injury.

It is generally said that the case fatality rate in the human infection is very high, 95 per cent or more, but recent serological evidence suggests that subclinical infection may be quite common, and the high mortality figure is appli-

cable only to clinical, untreated cases.[116] [126] The tetracyclines appear to be the most active of the chemotherapeutic agents when tested *in vitro*,[113] but the experimental disease in mice is most effectively treated with chloramphenicol or sulfonamides, the tetracyclines requiring extended periods of treatment to give 70 per cent or better cures.[69]

Transmission of the infection among guinea pigs by biting insects, mosquitoes and fleas, has been reported. Rodents usually die within a short time from septicemia, and the lesions appear as small nodules superficially resembling tubercles. Guinea pigs and rabbits are highly susceptible to inoculation. *A. whitmori* also produces the Straus reaction.

ACTINOBACILLOSIS

Actinobacillus Lignieresi. In 1902 Ligniers and Spitz isolated a nonmotile, nonbranching, non-acid-fast, gram-negative bacillus from the lesions of a disease of cattle which closely resembles actinomycosis and with which it is frequently confused. The microorganism is known as *A. lignieresi* and the disease as actinobacillosis. Originally found in Argentina, it appears to be relatively common and has been recognized in Europe and in this country.

Granules, very similar to but smaller and more numerous than the "sulfur granules" of actinomycosis, are found in the thick pus from the lesions. These granules or colonies contain clubs radially arranged about a center which is composed of detritus and gram-negative bacilli. The microorganism is pleomorphic in cultures; diplococci and slender rods are found in smears from cultures in liquid mediums while long curved forms are present in deep colonies in agar mediums. The bacilli are 0.4 μ in diameter and 1 to 15 μ in length.

Surface colonies on laboratory mediums are small, 0.5 to 1 mm. in diameter, smooth, glistening, convex, bluish white, and delicate in appearance. In liquid mediums, such as serum-glucose-broth, the growth consists of small grayish granules adhering to the sides of the tube and readily broken loose by shaking; broth does not become turbid. The microorganism appears to be a strict parasite and grows very poorly or not at all except in mediums containing serum or whole blood. It is an obligate aerobe though primary cultures are often more successful in fluid mediums or serum-glucose-agar stab cultures, especially when incubated in an atmosphere containing 10 per cent carbon dioxide. Cultures grow up in 24 hours at 37° C.; growth is very slight at 20° C. Dextrose, lactose, sucrose, maltose, raffinose, and mannitol are fermented; the fermentation of xylose is irregular. Arabinose, dulcitol, salicin, and inulin are not fermented. Very small amounts of indol are produced, coagulated serum is not liquefied, and litmus milk is usually unchanged but sometimes slightly acid.*

As indicated above, the disease in cattle closely resembles actinomycosis, differing in that the bones are seldom affected and the lesions are found in the soft tissues, the regional lymphatics being commonly involved; the subcutaneous tumors break down in time to form abscesses. The subcutaneous lesions respond to surgery but infection of the tongue, "wooden tongue," is often fatal. The disease has been observed to occur in both epizootic and sporadic form. It is readily reproduced by inoculation of cattle, but the microorganism is only feebly pathogenic for the usual experimental animals; massive intraperitoneal inoculation of the guinea pig produces a scrotal reaction similar to the Straus reaction. A very few probable cases of human infection have been reported.

Actinobacillus Actinoides. This microorganism was originally isolated from the lungs of calves suffering from chronic pneumonia and later from a similar affection in white rats. It is not pathogenic for other experimental animals, and, in fact, its etiological relationship to the observed disease is questionable.

Actinobacillus Actinomycetem-comitans. This bacillus has been observed and isolated from human cases of infec-

* There is some discrepancy in the literature on these points.

tion with *Actinomyces bovis*, first by Kligler in Germany in 1912, and by Comstock in England in 1920, and Bayne-Jones in 1925 in this country. It occurs as densely packed, gram-negative coccobacilli in contrast to the gram-positive actinomycete mycelium in the interior of the sulfur granules. It has never been reported in bovine actinomycosis, and the significance of its presence is open to question.

REFERENCES

1. Ajl, S. J., Reedal, J. S., Durrum, E. L., and Warren, J.: Studies on plague. I. Purification and properties of the toxin of *Pasteurella pestis*. J. Bact., 1955, **70:**158–169.
2. Ajl, S. J., and Rust, J. H.: The biochemistry and physiology of the plague murine toxin. Ann. N. Y. Acad. Sci., 1960, **88:**1152–1154.
3. Ajl, S. J., Rust, J. H., Hunter, D., Woebke, J., and Bent, D. F.: Preparation of serologically homogeneous plague murine toxin and its reactions with physical, chemical and enzymatic agents. J. Immunol., 1958, **80:**435–440.
4. Ajl, S. J., Woebke, J., and Rust, J. H.: Inhibition of keto acid oxidation by plague toxin. J. Bact., 1958, **75:**449–452.
5. Amies, C. R.: The envelope substance of *Pasteurella pestis*. Brit. J. Exptl. Path., 1951, **32:**259–273.
6. Baltazard, M.: Déclin et déstin d'une maladie infectieuse: la peste. Bull. W. H. O., 1960, **23:**247–262.
7. Baltazard, M., et al.: Recommended laboratory methods for the diagnosis of plague. Bull. W. H. O., 1956, **14:**457–509.
8. Baltazard, M., et al.: Recherches sur la peste en Iran. Bull. W. H. O., 1960, **23:**141–155.
9. Baltazard, M., and Bahmanyar, M.: Recherches sur la peste en Indie. Bull. W. H. O., 1960, **23:**169–215.
10. Baltazard, M., and Bahmanyar, M.: Recherches sur la peste à Java. Bull. W. H. O., 1960, **23:**217–246.
11. Baltazard, M., and Seydian, B.: Enquête sur les conditions de la peste au Moyen-Orient. Bull. W. H. O., 1960, **23:**157–167.
12. Beamer, P. R., Varney, P. L., Brown, W. G., and McDowell, F.: Studies on *Malleomyces pseudomallei* isolated from melioidosis originating in the western hemisphere. Amer. J. Clin. Path., 1954, **24:**1231–1240.
13. Bell, J. F., Owen, C. R., and Larson, C. L.: Virulence of *Bacterium tularense*. I. A study of the virulence of *Bacterium tularense* in mice, guinea pigs and rabbits. J. Infect. Dis., 1955, **97:**162–166.
14. Bent, D. F., et al.: Elemental and amino acid composition of purified plague toxin. Proc. Soc. Exptl. Biol. Med., 1957, **95:**178–181.
15. Bhagavan, N. V., Chen, T. H., and Meyer, K. F.: Further studies of antigenic structure of *Pasteurella pestis* in gels. Proc. Soc. Exptl. Biol. Med., 1956, **91:**353–356
16. Biberstein, E. L., and Cameron, H. S.: The family Brucellaceae in veterinary research. Ann. Rev. Microbiol., 1961, **15:**93–118.
17. Buckland, F. E., and Treadwell, R. H.: A comparison of plague vaccines by the mouse protection test. J. Hyg., 1961, **59:**49–56.
18. Burns, G. C. T., Espiner, E. A., and Perry, E. G.: "Cat bite" disease. Report of a case. New Zealand Med. J., 1959, **58:**598–599.
19. Burroughs, A. L., Holdenried, R., Longanecker, D. S., and Meyer, K. F.: A field study of latent tularemia in rodents with a list of all known naturally infected vertebrates. J. Infect. Dis., 1945, **76:**115–119.
20. Burrows, T. W.: The basis of virulence for mice of *Pasteurella pestis*. Society for General Microbiology, Symposium No. 5, pp. 151–175. Cambridge University Press, 1955.
21. Burrows, T. W.: Virulence of *Pasteurella pestis*. Nature, 1957, **179:**1246–1247.
22. Burrows, T. W.: Biochemical properties of virulent and avirulent strains of bacteria: *Salmonella typhosa* and *Pasteurella pestis*. Ann. N. Y. Acad. Sci., 1960, **88:**1125–1135.
23. Burrows, T. W., and Bacon, G. A.: The basis of virulence in *Pasteurella pestis:* Comparative behavior of virulent and avirulent strains in vivo. Brit. J. Exptl. Path., 1954, **35:**134–143.
24. Burrows, T. W., and Bacon, G. A.: The basis of virulence in *Pasteurella pestis:* An antigen determining virulence. Brit. J. Exptl. Path., 1956, **37:**481–493.
25. Burrows, T. W., and Bacon, G. A.: The effects of loss of different virulence determinants on the virulence and immunogenicity of strains of *Pasteurella pestis*. Brit. J. Exptl. Path., 1958, **39:**278–291.
26. Burrows, T. W., and Bacon, G. A.: V and W antigens in strains in *Pasteurella pseudotuberculosis*. Brit. J. Exptl. Path., 1960, **41:** 38–44.
27. Carter, G. R.: Some comments on pasteurellosis. Canadian J. Comp. Med. Vet. Sci., 1952, **16:**150–152.
28. Carter, G. R.: The type specific capsular antigen of *Pasteurella multocida*. Canadian J. Med. Sci., 1952, **30:**48–53.
29. Carter, G. R.: Studies on *Pasteurella multocida*. I. A hemagglutination test for the identification of serological types. Amer. J. Vet. Res., 1955, **16:**481–484.
30. Carter, G. R.: Studies on *Pasteurella multocida*. II. Identification of antigenic characteristics and colonial variants. Amer. J. Vet. Res., 1957, **18:**210–212.
31. Carter, G. R., and McSherry, B. J.: Further observations of shipping fever in Canada. Canadian J. Comp. Med. Vet. Sci., 1955, **19:**177–181.
32. Cavanaugh, D. C., and Randall, R.: The role of multiplication of *Pasteurella pestis* in mononuclear phagocytes in the pathogenesis of flea-borne plague. J. Immunol., 1959, **83:** 348–363.
33. Chen, T. H., Foster, L. E., and Meyer, K. F.: Experimental comparison of the immunogenicity of antigens in the residue of ultrasonated avirulent *Pasteurella pestis* with a vaccine prepared with killed virulent whole organisms. J. Immunol., 1961, **87:**64–71.
34. Chen, T. H., and Meyer, K. F.: Studies on immunization against plague. X. Specific precipitation of *Pasteurella pestis* antigens

and antibodies in gels. J. Immunol., 1955, **74:**501–507.
35. Cocking, E. C., Keppie, J., Witt, K., and Smith, H.: The chemical basis of the virulence of *Pasteurella pestis*. II. The toxicity for guinea pigs and mice of products of *Past. pestis*. Brit. J. Exptl. Path., 1960, **41:**460–471.
36. Coghlan, J. D.: Isolation of *Pasteurella multocida* from human peritoneal pus and a study of its relationship to other strains of the same species. J. Path. Bact., 1958, **76:**45–53.
37. Colling, M., Nigg, C., and Heckly, R. J.: Toxins of *Pseudomonas pseudomallei*. I. Production in vitro. J. Bact., 1958, **76:**422–426.
38. Cravitz, L., and Miller, W. R.: Immunologic studies with *M. mallei* and *M. pseudomallei*. II. Agglutination and complement fixation tests in man and laboratory animals. J. Infect. Dis., 1950, **86:**52–62.
39. Crocker, T. T., Chen, T. H., and Meyer, K. F.: Electron microscopic study of the extracellular materials of *Pasteurella pestis*. J. Bact., 1956, **72:**851–857.
40. Crumpton, M. J., and Davies, D. A. L.: An antigenic analysis of *Pasteurella pestis* by diffusion of antigens and antibodies in agar. Proc. Roy. Soc., Series B, 1956, **145:**109–134.
41. Crumpton, M. J., Davies, D. A. L., and Hutchinson, A. M.: The serological specificities of *Pasteurella pseudotuberculosis* somatic antigens. J. Gen. Microbiol., 1958, **18:**129–139.
42. Daniels, J. J. H. M.: Enteral infection with *Pasteurella pseudotuberculosis*. Isolation of the organism from human faeces. Brit. Med. J., 1961, **ii:**997.
43. Davies, D. A. L.: The smooth and rough somatic antigens of *Pasteurella pseudotuberculosis*. J. Gen. Microbiol., 1958, **18:**118–128.
44. Delwiche, E. A., Fukui, G. M., Andrews, A. W., and Surgalla, M. J.: Environmental conditions affecting the population dynamics and the retention of virulence of *Pasteurella pestis:* The role of carbon dioxide. J. Bact., 1959, **77:**355–360.
45. Devignat, R.: Variétés de l'espèce *Pasteurella pestis*. Nouvelle hypothèse. Bull. W. H. O. 1951, **4:**247–263.
46. Devignat, R.: Comportement biologique et biochimique de *P. pestis* et de *P. pseudotuberculosis*. Bull. W. H. O., 1954, **10:**463–494.
47. Ehrenkranz, N. J., and Meyer, K. F.: Studies on immunization against plague. VIII. Study of three immunizing preparations in protecting primates against pneumonic plague. J. Infect. Dis., 1955, **96:**138–144.
48. Ehrenkranz, N. J., and White, L. P.: Hepatic function and other physiologic studies in monkeys with experimental pneumonic plague. J. Infect., Dis., 1954, **95:**226–231.
49. Eigelsbach, H. T., Braun, W., and Herring, R. D.: Studies on the variation of *Bacterium tularense*. J. Bact., 1951, **61:**557–569.
50. Eigelsbach, H. T., Braun, W., and Herring, R. D.: Studies on the immunogenic properties of *Bacterium tularense* variants. J. Infect. Dis., 1952, **91:**86–91.
51. Eisler, D. M., Kubik, G., and Preston, H.: Colonial morphology and virulence of *Pasteurella pestis*. J. Bact., 1958, **76:**41–47.
52. Eisler, D. M., Kubik, G., and Preston, H.: Dissociation in *Pasteurella pestis*. I. Immunological comparisons of smooth and nonsmooth variants. J. Bact., 1958, **76:**589–596.
53. Eisler, D. M., Kubik, G., and Preston, H.: Dissociation in *Pasteurella pestis*. II. Interrelations of smooth and nonsmooth variants. J. Bact., 1958, **76:**597–606.
54. Elberg, S. S., and Ho, C.-L.: Studies on dissociation in *Pasteurella multocida*. J. Comp. Path. Therap., 1950, **60:**41–50.
55. Englesberg, E., et al.: Virulence in *Pasteurella pestis*. Science, 1954, **119:**413–414.
56. Englesberg, E., and Levy, J. B.: Production of *Pasteurella pestis* toxin. J. Bact., 1954, **68:**57–60.
57. Eskey, C. R., and Haas, V. H.: Plague in the western part of the United States. Public Health Bulletin No. 254. Public Health Service, U. S. Department of Health, Education and Welfare. 1940.
58. Fenyuk, B. K.: Experience in the eradication of enzootic plague in the north-west part of the Caspian region of the USSR. Bull. W. H. O., 1960, **23:**263–273.
59. Foshay, L.: Tularemia. Ann. Rev. Microbiol., 1950, **4:**313–330.
60. Fukui, G. M., et al.: The effect of temperature on the synthesis of virulence factors by *Pasteurella pestis*. Ann. N. Y. Acad. Sci., 1960, **88:**1146–1151.
61. Girard, G.: Dépistage post mortem de la peste par ponctions d'organes. Trente années d'expériences à Madagascar. Bull. W. H. O., 1952, **5:109–116.**
62. Girard, G.: Méthodes permettant de différencier *P. pestis* de *P. pseudotuberculosis*. Possibilité d'uniformiser ces méthodes. Bull. W. H. O., 1953, **9:**645–653.
63. Girard, G.: Les trois variétés du bacille pesteux (*Pasteurella pestis*). Intérét épidémiologique de cette notion. Maroc. Med., 1954, **33:**1016–1018.
64. Girard, G.: Plague. Ann. Rev. Microbiol., 1955, **9:**253–276.
65. Green, T. W., and Eigelsbach, H. T.: Immunity in tularemia. Report of two cases of proved reinfection. Arch. Intern. Med., 1950, **85:**777–782.
66. Heckly, J. R., and Nigg, C.: Toxins of *Pseudomonas pseudomallei*. II. Characterization. J. Bact., 1958, **76:**427–436.
67. Herbert, D.: Studies on the nutrition of *Pasteurella pestis*, and factors affecting the growth of isolated cells on an agar surface. Brit. J. Exptl. Path., 1949, **30:**509–519.
68. Hesselbrock, W., and Foshay, L.: The morphology of *Bacterium tularense*. J. Bact., 1945, **49:**209–231.
69. Hezebicks, M. M., and Nigg, C.: Chemotherapy of experimental melioidosis in mice. Antibiot. Chemotherapy, 1958, **8:**543–560.
70. Higuchi, K., and Carlin, C. E.: Studies on the nutrition and physiology of *Pasteurella pestis*. I. A casein hydrolysate medium for the growth of *Pasteurella pestis*. J. Bact., 1957, **73:**122–129.
71. Higuchi, K., and Carlin, C. E.: Studies on the nutrition and physiology of *Pasteurella pestis*. II. A defined medium for the growth of *Pasteurella pestis*. J. Bact., 1958, **75:**409–413.
72. Hirst, L. F.: The Conquest of Plague. A Study of the Evolution of Epidemiology. Oxford University Press, 1953.

73. Hoessly, G. F., Walker, D. L., Larson, A., and Meyer, K. F.: Experimental bubonic plague in monkeys. I. Study of the development of the disease and the peripheral circulatory failure. Acta Tropica, 1955, **12:**240–251.
74. Howe, C., and Miller, W. R.: Human glanders: Report of 6 cases. Ann. Intern. Med., 1947, **26:**93–105.
75. Jackson, S., and Burrows, T. W.: The pigmentation of *Pasteurella pestis* on a defined medium containing haemin. Brit. J. Exptl. Path., 1956, **37:**570–576.
76. Jackson, S., and Burrows, T. W.: The virulence-enhancing effect of iron on non-pigmented mutants of virulent strains of *Pasteurella pestis*. Brit. J. Exptl. Path., 1956, **37:**577–583.
77. Jackson, S., and Morris, B. C.: Enhancement of growth of *Pasteurella pestis* and other bacteria in serum by the addition of iron. Brit. J. Exptl. Path., 1961, **42:**363–368.
78. Jellison, W. L., and Kohls, G. M.: Tularemia in sheep and in sheep industry workers in western United States. Public Health Service Publication No. 421. Pub. Health Monograph No. 28. U. S. Department of Health, Education and Welfare. 1955.
79. Jellison, W. L., and Parker, R. R.: Rodents, rabbits and tularemia in North America. Some zoological and epidemiological considerations. Amer. J. Trop. Med., 1945, **25:** 349–362.
80. Kadull, P. J., Reames, H. R., Coriell, L. L., and Foshay, L.: Studies on tularemia. V. Immunization of man. J. Immunol., 1950, **65:** 425–435.
81. Kartman, L.: The role of rabbits in sylvatic plague epidemiology, with special attention to human cases in New Mexico and use of fluorescent antibody technic for detection of *Pasteurella pestis* in field specimens. Zoonoses Res., 1960, **1:**1–27.
82. Kartman, L., Miles, V. I., and Prince, F. M.: Ecological studies of wild rodent plague in the San Francisco Bay area of California. I. Introduction. Amer. J. Trop. Med. Hyg., 1958, **7:**112–124.
83. Kartman, L., Prince, F. M., Quan, S. F., and Stark, H. E.: New knowledge on the ecology of sylvatic plague. Ann. N. Y. Acad. Sci., 1958, **70:**668–711.
84. Kartman, L., Quan, S. F., and Miles, V. I.: Ecological studies of wild rodent plague in the San Francisco Bay area of California. V. The distribution of naturally occurring fleas during an epizootic in relation to their infection rates. Amer. J. Trop. Med. Hyg., 1960, **9:**96–100.
85. Keppie, J., Cocking, E. C., and Smith, H.: A non-toxic complex from *Pasteurella pestis* which immunizes both guinea pigs and mice. Lancet, 1958, **i:**246–247.
86. Keppie, J., Cocking, E. C., Witt, K., and Smith, H.: The chemical basis of the virulence of *Pasteurella pestis*. III. An immunogenic product obtained from *Past. pestis* which protects both guinea-pigs and mice. Brit. J. Exptl. Path., 1960, **41:**577–585.
87. Keppie, J., Smith, H., and Cocking, E. C.: Lethality for guinea pigs of ultrasonic extracts of *Pasteurella pestis:* Its relationship to death of guinea pigs from plague. Nature, 1957, **180:**1136.
88. Khaira, B. S., Young, W. B., and Hart, P. de V.: Melioidosis. Brit. Med. J., 1959, **i:** 949–952.
89. Knapp, W.: Die Agglutinationsreaktion und ihre Besonderheiten in der Serodiagnostik menschlicher Infektionen mit *Pasteurella pseudotuberculosis*. Ztschr. f. Hyg. u. Infektionskr., 1956, **143:**261–277.
90. Knapp, W.: Mesenteric adenitis due to *Pasteurella pseudotuberculosis* in young people. New England J. Med., 1958, **259:**776–778.
91. Korobkova, E. I.: Concerning the methods of increasing the immunogenic properties of various strains of plague vaccines and their stabilization. J. Microbiol. Epidemiol. Immunobiol., 1957, **28:**985–989. (Translated from the Russian.)
92. Landy, M., and Trapani, R. J.: A hemagglutination test for plague antibody with purified capsular antigen of *Pasteurella pestis*. Amer. J. Hyg., 1954, **59:**150–156.
93. Lapeyssonnie, L.: Mise au point: Bacille de Whitmore et melioidoses. Ann. Inst. Pasteur, 1958, **95:**334–342.
94. Levine, H. B., and Garber, E. D.: Detection of rough dissociants of *Pasteurella pestis* with tetrazolium chloride. J. Bact., 1950, **60:**508.
95. Levine, H. B., Lien, O. G., and Maurer, R. L.: Mortality enhancing polypeptide constituents from *Pseudomonas pseudomallei*. J. Immunol., 1959, **83:**468–477.
96. Lewis, M. L.: Fatal meningitis with septicemia caused by *Pasteurella multocida*. Amer. J. Clin. Path., 1953, **23:**241–245.
97. Link, V. B.: Plague. Amer. J. Trop. Med., 1951, **31:**452–457.
98. Link, V. B.: A history of plague in the United States of America. Public Health Service Publication No. 392. Public Health Monograph No. 26. U. S. Department of Health, Education and Welfare. 1955.
99. Lobel, L. W. M., Schaar, A. vd., and Roza, Moh.: Attenuation of *Bacillus mallei* with culture media containing ox-gall. Immunization tests with living avirulent bacilli in guinea-pigs and horses. Nederland Indische Blad. Diergeneesk., 1941, **53:**100–126.
100. Lusby, M., and Levine, H. B.: An intracellular mortality-enhancing material from *Malleomyces pseudomallei*. J. Immunol., 1958, **80:** 446–453.
101. Mager, J., Traub, A., and Grossowicz, N.: Cultivation of *Pasteurella tularensis* in chemically defined media: Effect of buffers and spermine. Nature, 1954, **174:**747–748.
102. Mathews, W. R.: Fatal tularemia with post mortem examination. New Orleans Med. Surg. J., 1938, **90:**479–488.
103. McCrumb, F. R., Jr., Larson, A., and Meyer, K. F.: The chemotherapy of experimental plague in the primate host. J. Infect. Dis., 1953, **92:**273–287.
104. Mendelson, R. W.: Glanders. U. S. Armed Forces Med. J., 1950, **1:**781–784.
105. van Metre, T. E., Jr., and Kadull, P. J.: Laboratory-acquired tularemia in vaccinated individuals: A report of 62 cases. Ann. Intern. Med., 1959, **50:**621–632.
106. Meyer, K. F.: Modern therapy of plague. J. Amer. Med. Assn., 1950, **144:**982–985.
107. Meyer, K. F.: Immunity in plague: A critical consideration of some recent studies. J. Immunol., 1950, **64:**139–163.

108. Meyer, K. F.: Recent studies on the immunity response to administration of different plague vaccines. Bull. W. H. O., 1953, **9:**619–636.
109. Meyer, K. F.: The natural history of plague and psittacosis. Pub. Health Repts., 1957, **72:** 705–719.
110. Meyer, K. F.: Pneumonic plague. Bact. Rev., 1961, **25:**249–261.
111. Miller, W. R., et al.: Studies on certain biological characteristics of *Malleomyces mallei* and *Malleomyces pseudomallei*. I. Morphology, cultivation, viability and isolation from contaminated specimens. J. Bact., 1948, **55:** 115–126.
112. Miller, W. R., et al.: Studies on certain biological characteristics of *Malleomyces mallei* and *Malleomyces pseudomallei*. II. Virulence and infectivity for animals. J. Bact., 1948, **55:** 127–135.
113. Moustardier, G., Dulong de Rosnay, C., and Salvat, J.: Étude de la sensibilité aux antibiotiques d'une souche de bacille de Whitmore. Ann. Inst. Pasteur, 1959, **96:**697–701.
114. Neel, R., and Girard, G.: Indications et limites de la réaction d'hémagglutination protéinique dans la peste. Bull. Soc. Path. Exot., 1955, **48:** 139–146.
115. Neel, R., Taslimi, H., and Eftekhari, M.: Valeur pratique comparée des réactions d'agglutination, de conglutination directe, d'hémagglutination polyosidique et protéinique pour le diagnostic de la peste. Rev. d'Immunol., 1954, **18:**87–106.
116. Nigg, C., and Johnston, M. M.: Complement fixation test in experimental clinical and subclinical melioidosis. J. Bact., 1961, **82:**159–168.
117. Ogg, J. E., Friedman, S. B., Andrews, A. W., and Surgalla, M. J.: Factors influencing the loss of virulence in *Pasteurella pestis*. J. Bact., 1958, **76:**185–191.
118. Packer, L., Rust, J. H., and Ajl, S. B.: Action of plague murine toxin on mammalian mitochondrial respiration. J. Bact., 1959, **78:**658–663.
119. Panja, G., and Chatterjee, B. C.: A case of acute cutaneous glanders in man. Indian Med. Gaz., 1943, **78:**150–151.
120. Parker, R. R., Steinhaus, E. A., Kohls, G. M., and Jellison, W. L.: Contamination of natural waters and mud with *Pasteurella tularensis* and tularemia in beavers and muskrats in the northwestern United States. National Institutes of Health Bull. No. 193. Public Health Service. U. S. Department of Health, Education and Welfare. 1951.
121. Philip, C. B., and Owen, C. R.: Comments on the nomenclature of the causative agent of tularemia. Internat. Bull. Bact. Nomen. Taxon., 1961, **11:**67–72.
122. Pirt, S. J., Thackery, E. J., and Harris-Smith, R.: The influence of environment on antigen production by *Pasteurella pestis* studied by means of the continuous flow culture technique. J. Gen. Microbiol., 1961, **25:**119–130.
123. Pollitzer, R.: Classification de la peste par des méthodes biochimiques. État des connaissances et orientation des recherches futures. Bull. W. H. O., 1953, **9:**655–664.
124. Pollitzer, R.: Plague. W. H. O. Monograph Series No. 22. World Health Organization, Palais des Nations, Geneva. 1954.
125. Pollitzer, R.: A review of recent literature on plague. Bull. W. H. O., 1960, **23:**313–400.
126. Prevatt, A. L., and Hunt, J. S.: Chronic systemic melioidosis. Review of the literature and report of a case with a note on visual disturbance due to chloramphenicol. Amer. J. Med., 1957, **23:**810–823.
127. Quan, S. F., McManus, A. G., and von Fintel, H.: Infectivity of tularemia applied to intact skin and ingested in drinking water. Science, 1956, **123:**942–943.
128. Ransom, J. P.: Some aspects of relationship between antigens of *Pasteurella pestis* and *Pasteurella pseudotuberculosis*. Proc. Soc. Exptl. Biol. Med., 1956, **93:**551–554.
129. Ransom, J. P., and Krueger, A. P.: Chronic pneumonic plague in *Macaca mulatta*. Amer. J. Trop. Med. Hyg., 1954, **3:**1040–1054.
130. Rapaport, F. T., Millar, J. W., and Ruch, J.: Endotoxic properties of *Pseudomonas pseudomallei*. Arch. Path., 1961, **71:**429–436.
131. Report: Expert Committee on Plague. Third Report. World Health Organization, Technical Rept. Ser. No. 165. Palais des Nations, Geneva. 1959.
132. Rockenmacher, M., James, H. A., and Elberg, S. S.: Studies on the nutrition and physiology of *Pasteurella pestis*. I. A chemically defined medium for *Pasteurella pestis*. J. Bact., 1952, **63:**785–794.
133. Saslaw, S., et al.: Tularemia vaccine study. Arch. Intern. Med., 1961, **107:**689–701; 702–714.
134. Schär, M., and Meyer, K. F.: Studies on immunization against plague. XV. The pathophysiologic action of the toxin of *Pasteurella pestis* in experimental animals. Schweiz. Z. allg. Path. Bakt., 1956, **19:**51–70.
135. Schär, M., and Thal, E.: Comparative studies on toxins of *Pasteurella pestis* and *Pasteurella pseudotuberculosis*. Proc. Soc. Exptl. Biol. Med., 1955, **88:**39–42.
136. Schmidt, J.: Über *Pasteurella pseudotuberculosis* Infektionen des Menschen. Arch. f. Hyg. u. Bakt., 1959, **143:**262–286.
137. Silverman, M. S., Elberg, S. S., Meyer, K. F., and Foster L.: Studies on immunization against plague. III. Quantitative serological studies on an immunizing antigen of *Pasteurella pestis*. J. Immunol., 1952, **68:**609–620.
138. Smith, H., Keppie, J., Cocking, E. C., and Witt, K.: The chemical basis of the virulence of *Pasteurella pestis*. I. The isolation and the aggressive properties of *Past. pestis* and its products from infected guinea pigs. Brit. J. Exptl. Path., 1960, **41:**452–459.
139. Smith, P. N.: Pneumonic plague in mice: Gross and histopathology in untreated and passively immunized animals. J. Infect. Dis., 1959, **104:** 78–84.
140. Sokhey, S. S., Habbu, M. K., and Bharucha, K. H.: Hydrolysate of casein for the preparation of plague and cholera vaccines. Bull. W. H. O., 1950, **3:**25–31.
141. Speck, R. S., and Wolochow, R.: Studies on the experimental epidemiology of respiratory infections. VIII. Experimental pneumonic plague in *Macacus rhesus*. J. Infect. Dis., 1957, **100:**58–69.
142. Surgalla, M. J.: Properties of virulent and avirulent strains of *Pasteurella pestis*. Ann. N. Y. Acad. Sci., 1960, **88:**1136–1145.
143. Talbot, J. M., and Sneath, P. H. A.: A taxonomic study of *Pasteurella septica*, especially of strains isolated from human sources. J. Gen. Microbiol., 1960, **22:**303–311.

144. Thal, E.: Relations immunologiques entre *Pasteurella pestis* et *Pasteurella pseudotuberculosis*. Ann. Inst. Pasteur, 1956, **91:**68–74.
145. Thal, E., and Chen, T. H.: Two simple tests for the differentiation of plague and pseudotuberculosis bacilli. J. Bact., 1955, **69:**103–104.
146. Trummert, W., and Remky, H.: Contribution à l'étude sérologique et thérapeutique des pasteurelloses humaines à *Past. multocida*. Presse Med., 1959, **67:**1925–1927.
147. Vest, E. D., and Marchette, N. J.: Transmission of *Pasteurella tularensis* among desert rodents through infective carcasses. Science, 1959, **128:**363–364.
148. Winter, C. C., Cherry, W. B., and Moody, M. D.: An unusual strain of *Pasteurella pestis* isolated from a fatal human case of plague. Bull. W. H. O., 1960, **23:**408–409.
149. Won, W. D.: New medium for the cultivation of *Pasteurella tularense*. J. Bact., 1958, **75:** 237–239.

Chapter 26

THE HEMOPHILIC BACTERIA

THE HEMOPHILIC bacteria are characterized by a nutritional requirement for constituents of fresh blood, especially hemoglobin and certain related compounds, or the X factor, and/or a requirement for the heat-labile V factor which may be replaced by DPN or TPN. Not all bacteria grouped under the genus Hemophilus have these nutritive requirements, notably the pertussis bacillus, the Morax-Axenfeld bacillus and Ducrey's bacillus, but they are otherwise similar. They are related antigenically to certain of the Brucella species, such as *Br. bronchiseptica* as noted elsewhere and lines of distinction may be difficult to draw. The pertussis bacillus has, in fact, been placed in a separate genus, Bordetella, by some.

The Hemophilic Group

SPECIES	GROWTH REQUIRMENTS		HEMOLYSIS
	X Factor	V Factor	
H. influenzae	+	+	−
H. hemolyticus	+	+	+
H. parainfluenzae	−	+	±
H. suis (*influenzae suis*)	+	+	−
H. canis (*hemoglobinophilus*)	+	−	−
H. pertussis	−	−	+
H. duplex (Morax-Axenfeld)	−	−	±
H. ducreyi	−	−	+

HEMOPHILUS INFLUENZAE (PFEIFFER'S BACILLUS)

Hemophilus influenzae was isolated by Pfeiffer in 1892, and until the early 1930's was regarded by many as the etiological agent of epidemic influenza. Influenza has, however, been shown to be caused by a filterable virus, and the name influenzae has no etiological significance.

Morphology and Staining. Pfeiffer's bacillus is one of the smallest pathogenic bacteria, rarely exceeding 1.5 μ in length and 0.3 μ in thickness. The ends of the cell are rounded, capsules are not generally observed but are present in smooth cultures, spores are not formed, and the bacillus is nonmotile. There is a marked tendency to produce threads and other anomalous forms in culture which is, to some degree, a characteristic of strains. Some workers have attempted to differentiate varieties on the basis of morphology, but there is a continuous series of types, ranging from predominantly coccobacillary forms to predominantly longer bacilli and threads, and no sharp distinction can be made. There is a tendency, however, to regard the coccobacillary forms as "typical" and the longer forms as "atypical"; the typical form appears to predominate in strains isolated from pathological processes.

On blood agar the colonies of Pfeiffer's bacillus are very small, rounded, discrete and transparent and may reach the size of a small pinhead. If the culture is con-

Figure 136. *Hemophilus influenzae*, pure culture. Note the variability from coccoid to bacillary form and the presence of longer filaments. Fuchsin; × 1050.

taminated with other microorganisms, especially *Staphylococcus aureus*, the colonies are considerably larger, more opaque, and of a grayish white color, and develop most luxuriantly in the neighborhood of the foreign colony, a phenomenon termed the satellite phenomenon.

These bacilli are somewhat more difficult to stain than most bacteria; Löffler's methylene blue for 5 minutes or dilute (1:10) carbol-fuchsin for 10 minutes is satisfactory. They are gram-negative.

Physiology. One of the more fastidious bacteria, Pfeiffer's bacillus requires, as noted above, the presence of blood in the culture medium. It has been found that two substances present in such blood mediums are necessary to the growth of this bacterium; one, designated as the "X factor," is heat-stable and associated with hemoglobin, and the other, the "V factor," is heat-labile and is found in yeast and various vegetable extracts as well as in whole blood. The satellite phenomenon noted above is due to the formation of the V factor by other bacteria and its diffusion into the medium from the colony.

The X factor is replaceable by hemoglobin or hematin. The iron protoporphyrin can be replaced with certain other iron porphyrins, but not by porphyrins such as meso-, hemato-, deutero-, and coproporphyrins. These last suppress growth by a competitive inhibition,[20] and such activity is eliminated by methylation of the propionic acid side chains.[21] It seems that these or related compounds are required for respiration —some strains do not require it for anaerobic growth. It has been suggested that hematin is required for the synthesis of catalase; hematin can be replaced by cysteine which would reduce peroxide and make catalase unnecessary.[17]

The V factor may be replaced by coenzyme I or coenzyme II but not by nicotinic acid or its amide. Apparently the whole coenzyme molecule must be supplied, and it is assumed that the thermolabile substance is of this nature. Some strains require 1,4-diaminobutane, the active residue available in putrescine, spermine, and spermidine.

A number of mediums have been devised for the cultivation of the influenza bacillus. Avery's oleate hemoglobin agar, prepared by the addition of sodium oleate and a suspension of erythrocytes to an infusion agar base, is one of the best of these, for not only is the growth of *H. influenzae* enhanced, but the growth of streptococci and pneumococci and some other bacteria present in the sputum and nasal mucus is inhibited. The influenza bacillus grows luxuriantly on chocolate agar, prepared by the addition of fresh blood to hot (90° C.) infusion agar, and heavy growths for agglutination and other purposes may be obtained, but this medium does not differentiate

and hence is not especially suitable for primary isolation. Fildes' agar, an infusion agar base to which has been added a peptic digest of blood, supports good growth of the influenza bacillus and is used especially by British workers.

No growth occurs on gelatin. Milk, containing blood, is rendered slightly alkaline by some strains. Nitrate is reduced to nitrite. Some strains—about 50 per cent—form indol. Fermentation reactions are variable, some strains being inactive while others ferment dextrose and other carbohydrates. Some strains are hemolytic while others are not. The hemolytic strains of *H. influenzae* do not appear to be clearly marked from the nonhemolytic strains by any other differential characters, since indol production and carbohydrate fermentation occur in both groups.

The influenza bacillus shows little resistance toward external conditions. It is readily killed by drying and pure culture suspended in water and then dried on silk threads dies within 24 hours but in dried sputum viability persists somewhat longer, but not, as a rule, beyond 48 hours. The bacilli are readily killed by disinfectants. Even under favorable conditions artificial cultures soon die out, and, in order to preserve vitality, subcultures must be made every 4 or 5 days; chocolate agar is suitable for maintaining stock cultures.

Varieties. Subdivisions of *H. influenzae* have been made by a number of workers, but whether such varieties deserve the dignity of species standing is open to question. The morphologically "typical" and "atypical" forms have been noted above, but these are not regarded as separate species. Other distinctions which have been made on the basis of hemolysis and nutritive requirements may be summarized briefly:

(1) The hemolytic and nonhemolytic varieties. The nonhemolytic form is designated *H. influenzae* by Bergey and the hemolytic form *H. hemolyticus*. Both require the X and V nutritive factors. These are generally regarded as a single species, *H. influenzae*.

(2) The swine influenza bacillus, *H. influenzae suis* or *H. suis*, which closely resembles Pfeiffer's bacillus except that it is relatively inert biochemically and differs immunologically. This bacterium, in association with a filterable virus, is causally associated with swine influenza. Both X and V factors are required for growth.

(3) The para-influenza bacilli, *H. para-influenzae*, which closely resemble *H. influenzae* except that only the V factor is required for growth and these bacteria may be cultivated on agar containing serum or ascitic fluid. Although these bacilli are defined as nonhemolytic, hemolytic strains showing the same nutritive requirements are found.

(4) *H. canis* (*H. hemoglobinophilus, H. hemoglobinophilus canis*), found in the preputial secretions of dogs. It closely resembles *H. influenzae* except that it requires only the X factor for growth.

Variation and Antigenic Structure. As tested by direct agglutination, *H. influenzae* is antigenically heterogeneous, and rough and smooth forms occur in which the smooth form is encapsulated. By means of the agglutination test the encapsulated bacilli are found to fall into six immunological types, designated a, b, c, d, e, and f, and the antigens responsible are specific polysaccharides.[29] One of these, the type-specific substance of type b, is a polymer of ribose phosphate with a structure analogous to that of pentose nucleic acids in which the purine and pyrimidine portions are replaced by a second polyribose phosphate chain in 1:1′ glycosidic linkage.[52] Type e may occur as two subtypes, the one as occasional freshly isolated strains, and the other in stock strains.[51] Diagnostic antiserums may be prepared for each of these types.[1] Typing may be carried out by agglutination, by specific capsular swelling in the presence of homologous antibody, or as a precipitin reaction using phenol-extracted polysaccharide as the antigen.[28]

It appears that many strains isolated are in the rough form, and these, by the agglutination test, are immunologically heterogeneous. Antigenic proteins may be extracted from the cell substance of influenza bacilli. One of these, fraction M, was found to be common to most strains of *H. influenzae*, indicating an immunological homogeneity demonstrable by precipitin tests with appropriately prepared antigens. It will be

clear that the antigenic structure of *H. influenzae* is by no means fully understood as yet.

The influenza bacilli appear to be immunologically related to certain of the pneumococcus types; type a shows cross-reactions with pneumococcus type 6b and type b cross-reacts with pneumococcus type 6 and type 29.

The S-R dissociation is to some degree reversible by growth in the presence of anti-R immune serum. The relation of virulence to this dissociative change is not known.

Toxins. As in the case of many other bacteria, the cell substance of the influenza bacillus is toxic to experimental animals, mice in particular, upon parenteral inoculation. Toxic substances are produced in fluid cultures, are filterable, and may appear in appreciable quantities after 6 to 8 hours' incubation. Relatively large quantities of the filtrate (2 to 4 ml.) are necessary to produce death in rabbits, and it is probable that a true exotoxin is not formed.

Pathogenicity for Man. The pathogenicity of Pfeiffer's bacillus for man is shown by the occurrence of cases of meningitis, mostly in infants and of a high fatality, in which *H. influenzae* is found in pure culture in the cerebrospinal fluid. These cases of "influenzal meningitis," while not very numerous, show that certain strains of this microorganism possess definite pathogenicity. *H. influenzae* meningitis is the fourth commonest form of purulent meningitis; it occurs most often in the second 6 months of life, and the case fatality rate in untreated cases is between 90 and 100 per cent.[26] In one series of 758 cases of meningitis occurring between 1930 and 1953, *H. influenzae* type b was the causal agent in 128.[5, 8, 30] Occasional cases of otitis media, appendicitis, sinusitis, and other localized infections may be caused by this bacterium.

The influenza bacillus is frequently present in infections of the respiratory tract and is found on autopsy in pneumonic lesions under conditions where its pathogenicity is clearly indicated. It has also been associated with respiratory disease in premature infants,[11] and serological studies suggest that it may be a factor in chronic bronchitis.[18] Whether it is present as a primary or secondary invader in these cases is more uncertain. Its common occurrence in disease like measles, whooping cough, and tuberculosis indicates that its growth on human tissue is favored by the presence of other infecting agents. It seems probable that in respiratory infections *H. influenzae* is not the primary agent.

The relation of this bacillus to influenza is very likely that of a secondary invader to the initial virus infection. It has been found that in double infections of the chick embryo with influenza virus, the pathogenicity of *H. influenzae* is enhanced though that of the virus is not affected.[6] It is frequently but not invariably present in cases of influenza and, of course, occurs in the absence of this disease. In the case of swine influenza, the influenza bacillus is, in association with a filterable virus, causally related to the disease.

Chemotherapy. Chemotherapy has been of greatest interest in connection with *H. influenzae* meningitis which, in untreated cases, is almost always fatal. Penicillin is not effective, but sulfonamides, streptomycin, and chlortetracycline, sometimes in combination with antiserum, may be highly effective. In one series of cases studied,[26] for example, treatment with sulfonamide alone gave 15 per cent survival and in combination with antiserum 83 per cent survival, but complications including mental retardation, blindness, hydrocephalus, and convulsions occurred in 20 per cent of survivors. The rise in recovery rate, due to more effective therapy, has resulted in an increase in such complications, *i.e.*, in patients who would otherwise have died. A combination of sulfonamide, streptomycin, and antiserum gave a case fatality rate of 7.4 per cent, and of chlortetracycline and sulfonamide 4.3 per cent, but with the same incidence of complications. Treatment with sulfonamide, streptomycin, and chlortetracycline, occasionally reinforced with chloramphenicol, reduced the case fa-

tality rate to 3.2 per cent and the incidence of complications in survivors to 5 per cent. *H. influenzae* is not sensitive to penicillin, and in fact the first use to which this antibiotic was put was that of a selective agent incorporated in isolation mediums.

Pathogenicity for Lower Animals. Except in swine influenza, the influenza bacillus is probably not a natural pathogen of lower animals. Upon intraperitoneal inoculation into laboratory animals, mice, guinea pigs, and rabbits, large doses of these bacteria produce death within 1 or 2 days. Whether this is an actual invasion rather than a toxemia is uncertain; the bacilli may be found in the peritoneal exudate but usually not in the heart's blood, and petechial hemorrhages may be observed scattered over the peritoneum and, sometimes, the pleura. Certain strains produce a fatal infection in mice on intracerebral inoculation. As tested by intraperitoneal inoculation, the virulence of *H. influenzae* varies greatly from strain to strain; the virulent strains are in a minority, and strains from influenzal meningitis are generally among the more virulent.

Other hemophilic bacteria have been described in connection with diseases of lower animals. *H. canis,* for example, has been found in association with inflammation of the prepuce in dogs but is apparently harmless. Other hemophilic bacilli such as *H. bovis, H. gallinarium, H. muris,* and *H. ovis* have been isolated from lower animals. Their relationship to *H. influenzae* and other better-known species is not clear.

THE KOCH-WEEKS BACILLUS

A small bacillus, first observed by Koch in 1883 in a series of eye inflammations in Egypt, was successfully cultivated by Weeks in New York in 1887, and is now recognized as the cause of a world-wide and highly contagious form of conjunctivitis sometimes known as pink-eye. The disease is especially prone to occur in tropical and subtropical climates, and may assume epidemic form.[45]

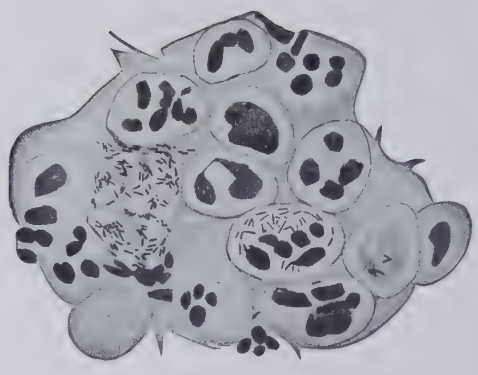


Figure 137. Koch-Weeks bacillus in conjunctivitis: × 900. (Axenfeld, Kolle, and Wassermann.)

This microorganism is a gram-negative, nonmotile, nonencapsulated facultative aerobe, and sometimes shows bipolar staining. It requires the X and V factors for growth. There is no hemolysis on blood agar, and the colonies are small and translucent with a bluish tinge in transmitted light.

This bacillus is closely related to *H. influenzae* and has been regarded by many as identical with it. Pittman and Davis[35] have found, however, that strains of this organism make up a closely related but not homogeneous serological group, distinct from the heterogeneous non-type-specific *H. influenzae,* and differentiate it as *H. aegyptius.* Antigenic components are, however, shared with *H. influenzae,* and are demonstrable by the gel-diffusion method.[33]

It produces conjunctivitis in man but not in laboratory animals. It has low virulence for mice when inoculated in mucin suspension, a high virulence for the 8-day chick embryo, but not for the 12-day embryo.

HEMOPHILUS PERTUSSIS (BORDETELLA PERTUSSIS)

Bacilli resembling *H. influenzae* were reported by early observers as occurring in a large proportion of cases of whooping cough. Although there are minor differences in the descriptions of these

Figure 138. *Hemophilus pertussis,* pure culture. Fuchsin; × 1050.

organisms as given by different observers, the cultural and morphological characters are essentially similar, and there seems little doubt that Spengler, Jochmann and Kraus, Wollstein, and Davis discovered the same bacillus. More definite results were obtained by Bordet and Gengou in 1908, who found in the bronchial exudate from patients of whooping cough a characteristic short oval bacillus which grew feebly on a special medium they devised. Earlier named *Bacillus pertussis,* this microorganism is now known either by that name or as *H. pertussis,* or, more casually, as the Bordet-Gengou bacillus.

Morphology and Staining. The Bordet-Gengou bacillus is a small ovoid rod from 1.0 to 1.5 μ in length and 0.3 to 0.5 μ in breadth. The majority of the bacteria occur singly, although they may occasionally be seen in pairs end to end; chains do not occur in smears of bronchial exudate, but short chains may be seen in cultures in liquid mediums. This morphology is relatively constant, and there is not the tendency to the formation of thread-like and other aberrant forms which is exhibited by the influenza bacillus. *H. pertussis* is nonmotile and non-spore-forming, and the smooth form is encapsulated.

On the Bordet-Gengou medium the colonies are smooth, raised, and glistening, with a metallic or pearl-like luster, and are larger and more opaque than those of the influenza bacillus; 48 to 72 hours' incubation is required for their appearance and development. Upon further incubation they acquire a slight brownish color. A mucoid substance is abundantly produced by the culture, and the growth is sticky and tenacious. On blood agar the colonies are surrounded by a narrow zone of hazy hemolysis.

The bacilli stain with a little difficulty and, as in the case of the influenza bacillus, methylene blue or dilute carbol-fuchsin must be applied for 5 to 10 minutes; carbol toluidine blue is recommended by some workers and stains the bacilli a lilac color. A tendency to bipolar staining may be observed. They are gram-negative.

Physiology. *H. pertussis* is difficult to cultivate upon primary isolation, and the Bordet-Gengou medium upon which it grows readily consists of 1 per cent glycerol agar or glycerol broth made with macerated potato and added to an equal volume of human or rabbit blood. By repeated passage on mediums containing less and less blood, however, it may be acclimatized and will, in time, grow, although sparsely, upon ordinary nutrient agar. It does not, therefore, require the V and X factors that are essential to the development of the hemophilic bacteria. The optimum temperature is 37° C., and the bacillus is aerobic and facultatively anaerobic.

Growth with the maintenance of immunogenic potency of phase I (see below) for vaccine production is a matter

of practical importance. A number of casein hydrolysate mediums have been devised[46, 49] for this purpose, and the inclusion of charcoal in beef heart agar tends to preserve immunogenic potency.[32] Starch is required in such mediums.

H. pertussis is biochemically inactive. It does not form indol, does not reduce nitrates, and does not ferment any sugars. Its resistance is slight and of the same order as that of the influenza bacillus. It is killed by exposure to 55° C. for 30 minutes.

Toxins. As in the case of Pfeiffer's bacillus, the cell substance of *H. pertussis* is toxic upon parenteral injection into experimental animals and to about the same degree. Endotoxin may be separated as a watery extract of disintegrated cells. There appear to be two fractions, one heat-stable and the other heat-labile and inactivated in 30 minutes at 56° C. On intravenous inoculation in rabbits the heat-stable fraction produces hyperglycemia and the heat-labile fraction hypoglycemia. Intratracheal inoculation in rabbits produces an edematous reaction followed by a lymphocytic infiltration about the blood vessels and bronchi which is reported to be similar to the changes produced in the lung in whooping cough. The endotoxin of a variant strain has been prepared by fractionation of sonic lysates[48] and by extraction of lyophilized bacilli with calcium chloride, followed by methanol precipitation in the cold, and contains about 5000 mouse LD_{50}/mg. N.[40]

Variation and Antigenic Structure. Unlike the influenza bacillus, *H. pertussis* is generally in the smooth state when isolated from the body on an optimal medium and is immunologically homogeneous, *i.e.*, the virulent form occurs only as a single antigenic type,[47] phase I. Culture strains fall into four immunological groups which are designated phases I, II, III, and IV. Although not obviously rough, phases III and IV are somewhat rougher in appearance and less stable in saline suspensions than the bacilli of phase I. The S-R dissociation of *H. pertussis* occurs readily on culture on artificial mediums, even on blood agar, and it seems probable that these phases do not represent distinct immunological types but rather successive stages in the S-R transformation. As indicated above, virulence and immunogenicity may be maintained by cultivation on charcoal agar.[36] The importance of the existence of the bacilli in phase I for the preparation of vaccines and the like has been stressed by many workers in the last few years; in all probability this is but another way of saying that the bacilli must be in the smooth state.

The S-R change, of which the above phases represent the early stages, proceeds to the obviously rough stage with consequent alterations in colonial morphology and loss of virulence.

As indicated elsewhere, *H. pertussis* is immunologically related to *Brucella bronchiseptica.* Atypical bacilli isolated from a small proportion of cases of whooping cough have been designated *B. para-pertussis.* These bacilli differ from *H. pertussis* in that they grow readily on ordinary nutrient agar upon isolation and produce an alkalinity in litmus milk in 2 to 4 days. Immunologically, they are related to both *H. pertussis* and *Br. bronchiseptica* through common O antigens, but are differentiated by capsular, or K, antigens.[2, 24]

Pathogenicity for Man.[23, 44] Whooping cough is widespread throughout the entire world, and it is estimated that 95 per cent of the population suffer from it, in typical or atypical form, at one time or another. The typical disease is commonest in the lower age groups and is much more serious in children than in adults. While the mortality has been steadily reduced, its relative importance as a cause of death under 5 years has increased progressively, and now it is exceeded in importance in this age group only by gastroenteritis, pneumonia and bronchitis, and tuberculosis. It occurs in three stages, each lasting about 2 weeks. The catarrhal stage begins with a mild cough that progresses in severity to a paroxysmal stage characterized by rapid consecutive coughs and the deep inspiratory whoop. In the convalescent

stage the number and frequency of paroxysms gradually decrease, and recovery is uneventful except that bronchial and lobar pneumonia and otitis media occur with some frequency as complications, especially in children. The bacilli are present in large numbers in the respiratory tract in the catarrhal, and most contagious, stage, and gradually disappear until they are rarely found after the fourth week.

Although certain features of whooping cough may be approximately reproduced in experimental animals, the mechanism by which the microorganisms produce the disease in man is not altogether clear. The characteristic lymphocytosis and the leucocytic exudate into the lumen of the trachea and bronchi have been regarded as evidence of the activity of the endotoxins. The bacilli are also present in large numbers, especially in the catarrhal stage, between the cilia and possibly interfere mechanically with ciliary action. The localization of the bacteria and especially the persistence of the paroxysmal cough after the bacteria are no longer detectable, however, tend to set whooping cough somewhat apart from the other bacterial respiratory infections. The observation that in the mouse either parenteral inoculation with vaccine or intranasal infection results in a remarkable increase in sensitivity to histamine (see below) suggests that some form of tissue sensitivity, of which increased histamine sensitivity may be a manifestation, plays a part in the pathogenesis of the disease.

Pathogenicity for Animals. Naturally occurring infections of lower animals with *H. pertussis* do not occur and, in general, it is of variable and usually low virulence for experimental animals. The developing chick embryo may be infected, with reproduction of the cellular infiltration and necrosis of the bronchi seen at autopsy of fatal cases of the human disease[41] and the bacilli may be grown in tissue culture.[7] Both the rat and the mouse may be infected by intranasal inoculation to produce interstitial pneumonia with leucocytic infiltration around the vessels and bronchioles and mucous secretion on the bronchial epithelium.[42] The mouse may be infected by intracerebral inoculation; smooth strains are the more virulent and the most virulent strains have an LD_{50} of 100 to 1000 bacilli by this route. Intracerebral infection of the mouse is used routinely in the standardization of the immunogenic potency of vaccines in this country; while it would appear to be quite unrelated to the human disease, Pittman[34] has found that there is a reasonable correlation between virulence as tested by this method and by intranasal inoculation of the mouse.

Bacteriological Diagnosis.[27] The bacillus may be isolated by the cough plate method in which the open plate is held 4 to 5 inches from the mouth and exposed to one or more explosive coughs. Nasopharyngeal swab cultures give a higher proportion of positive cultures than the cough-plate method, and the pernasal swab a higher proportion of positive cultures than the postnasal swab.[31] The swab, on thin, flexible, copper wire, is passed through a nostril into the nasopharynx and left there for two or three coughs before withdrawal, and cultured on Bordet-Gengou medium. Sputum culture is not particularly satisfactory. Colonies of *H. pertussis* are larger and more opaque than those of the influenza bacillus; it may be differentiated further by hemolysis on blood agar and growth in the absence of the X and V factors.

Chemotherapy.[22, 37] The chemotherapy of whooping cough is not as successful as that of many of the other

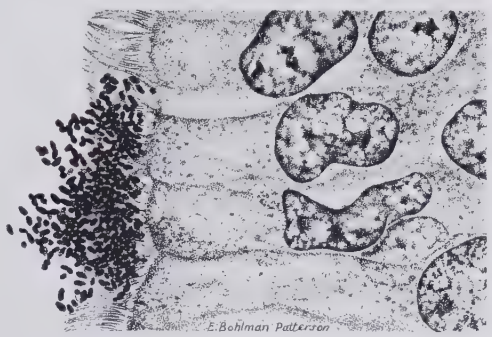


Figure 139. Whooping cough. Minute bacilli present in masses between cilia of two cells lining the trachea; × about 1500.

infectious diseases, but the broad spectrum antibiotics, chloramphenicol and the tetracyclines, have been found to reduce the duration and severity of the disease significantly in many instances, and occasional dramatic results have been reported. The sulfonamides and penicillin are ineffective.

Epidemiology.[19] An upper respiratory infection, whooping cough is transmitted by means of the secretions of the mouth and nose, to some extent through towels, handkerchiefs, hand-to-hand contact and the like, and undoubtedly to a great extent by droplet infection. Undiagnosed and atypical cases may play an important part in the dissemination of the disease. The age incidence is marked, with 96 per cent of the deaths in children under 5 years of age, and there is no doubt that whooping cough is one of the most important killing diseases of childhood. There appears to be some racial difference in susceptibility since the Negro death rate is considerably higher than that of the whites. In contrast to most other diseases the incidence in females is somewhat higher than that in males and in children in higher economic groups. The seasonal incidence shows a drop during late summer and early fall, with a prolonged peak in the later winter and spring. The disease tends to recur in periodic epidemic waves, presumably a consequence of the accumulation of a new crop of susceptibles.

Immunity. Recovery from whooping cough is accompanied by the development of immunity. Second attacks occur only infrequently in children and then are very mild; in older people second attacks are more severe. Complement-fixing antibodies are formed but do not appear until the third or fourth week of the disease and, consequently, are of limited diagnostic value.

Under experimental conditions there are three kinds of response to the bacilli. There is a very rapid development, usually within a few hours, of a refractory state in which the animals are highly, and specifically, resistant to challenge inoculation, which persists for a week or more[14, 15]; it is considered to be in the nature of an interference phenomenon. Second, as a consequence of inoculation with vaccine, or infection, mice become extremely sensitive to the action of histamine,[25] and it has been found that the factor(s) responsible is separable from the antigens eliciting the conventional immune response.[10] Finally, the usual antibody response, with the presence of protective, complement-fixing, etc., antibody demonstrable in the serum occurs also. Presumably only the last is concerned with effective prophylactic immunity.

Prophylactic inoculation. The early attempts to use vaccines of *H. pertussis* were not uniformly successful. More encouraging results were obtained by Madsen and other Danish bacteriologists in the Faroe Islands. In the United States Sauer and his colleagues have prepared vaccines of freshly isolated, virulent strains which appear to be effective in reducing the incidence and severity of the disease. It is of primary importance that the vaccine consist of the smooth bacilli of phase I, and earlier reports of the inefficacy of active immunization are probably attributable in part, at least, to the use of other than the smooth form of the microorganism. Immunogenic potency of vaccines requires constant assay and control; in the United States a standard test, using intracerebral challenge in the mouse, is used, and similar control procedures are used by others.[3] Alum-precipitated vaccine seems to be effective also, but it is not known whether it is superior to plain vaccine.

Pertussis vaccine may be successfully combined with diphtheria toxoid to allow immunization against the two diseases simultaneously; available evidence indicates that the immune response is equal to that obtained with separate antigens. It is generally agreed that the disease is appreciably milder in immunized children.

With the demonstration of the endotoxins of *H. pertussis*, considerable interest has attached to the possible role of anti-endotoxin in immunity to the disease. The toxicity appears to be neutral-

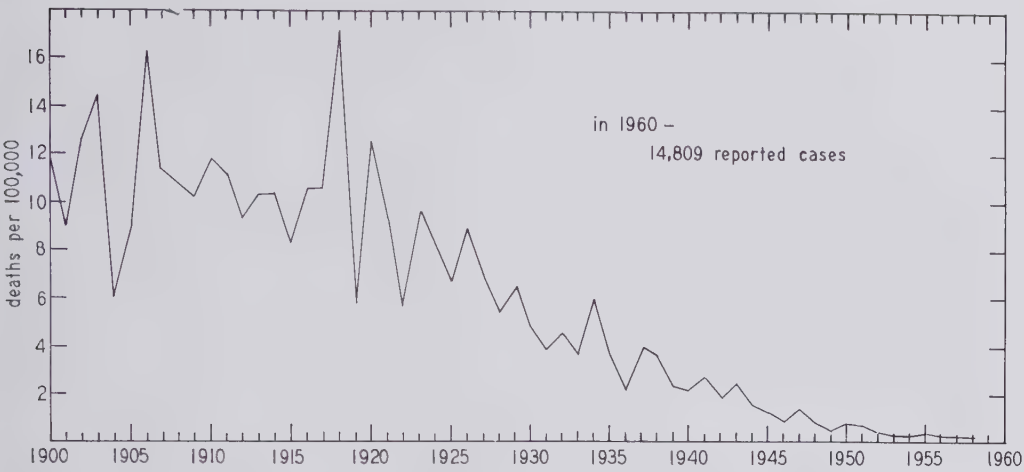


Figure 140. The prevalence of whooping cough as shown by the mortality rates in the Registration Area since 1900. Data from the Morbidity and Mortality Weekly Report, vol. 9, 1961. Communicable Disease Center, Public Health Service, U. S. Department of Health, Education and Welfare.

ized with appropriately prepared antiserum, and studies with experimental animals suggest that antitoxic antibody may be significant in the immunity.

THE MORAX-AXENFELD DIPLOBACILLUS (HEMOPHILUS DUPLEX)

A small bacillus, described independently by Morax and by Axenfeld in 1896 and 1897, is responsible for infections of the conjunctiva and cornea in man and is known as the Morax-Axenfeld bacillus or *B. lacunatus* (from the lacunae of liquefaction produced by growth on coagulated serum). It has been grouped with the hemophilic bacteria as *H. duplex.*

The short rods, 1 μ by 2 to 3 μ, frequently appear end to end in pairs or short chains. They are nonmotile, nonspore-forming and gram-negative. They do not grow on the ordinary nutrient mediums, potato, milk, or gelatin but require the presence of serum, ascitic fluid, or blood in the culture medium. They ferment few if any carbohydrates and do not form indol. Coagulated serum is liquefied. They die out within a day or two at room temperature but may survive for weeks in culture in the incubator. It has been reported that hemolytic and nonhemolytic types occur which may be differentiated immunologically.

So far as is known this microorganism is pathogenic only for the human eye. The inoculation of experimental animals is without effect, but the instillation of the bacilli onto the conjunctival sac of man results in the development of blepharo-conjunctivitis, either chronic or acute, and severe inflammation of the cornea may be produced. Treatment with 0.25 per cent zinc sulfate solution is specific and produces a rapid cure, while silver salts are without effect. Chlortetracycline is reported to be highly effective when used as a 1 per cent ointment, and streptomycin is effective also. The disease is widely distributed and has been reported in Europe, Africa, and North America.

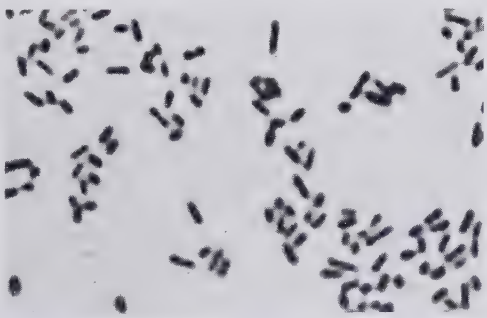


Figure 141. *Hemophilus duplex* (Morax-Axenfeld bacillus); pure culture. Gram stain; × 2400.

DUCREY'S BACILLUS (HEMOPHILUS DUCREYI)

Soft chancre or chancroid is a veneral disease transmitted by direct contact. The lesions, which are on the genitals or adjacent areas, are irregular ulcers which differ from the hard or Hunterian chancre —the primary lesion of syphilis—in that they are not indurated. Unlike syphilis, the infection remains localized, spreading no further than the neighboring lymphatics, which may become swollen to form secondary buboes in the groin.[4]

The bacillus was found by Ducrey in 1890 in the purulent discharge from the lesion, and by the inoculation of the skin of the forearm he was able to transmit the disease through fifteen generations. The microorganism was obtained in pure culture by Besançon, Griffon and le Sourd in the same year.

Ducrey's bacillus is a short rod 1 to 1.5 μ in length and 0.6 μ in breadth. In smears it is generally found to be ovoid, and there is a tendency to occur in end-to-end pairs or in short chains; in broth culture longer chains may be observed. It is non-spore-forming and nonmotile. The bacillus not infrequently stains irregularly and bipolar staining may be observed. It is stained by the usual aniline dyes and is gram-negative.

The bacillus will not grow on the ordinary laboratory mediums and requires the addition of serum, or, preferably, blood.[38] The X and V factors alone are not sufficent, and additional factors present in either serum or erythocytes are required. The small, grayish, glistening colonies appear in blood agar in 24 hours and, after 2 to 3 days' incubation, show a narrow zone of hemolysis. The bacilli may be cultivated from the chancroid by inoculating tubes of fresh (not more than 3 to 5 days old) rabbit blood. Smears made at the end of 24 to 48 hours' incubation will show the characteristic tangled chains of gram-negative bacilli. These bacteria cannot be identified in smears from the chancroid because of a tendency to aberrant morphology. The bacilli may be isolated and cultivated on the chorioallantois of the developing chick embryo and appear to have little pathogenicity for the embryo. Rabbits and monkeys may be infected by intradermal inoculation of pure cultures, but subcutaneous, intraperitoneal, and intravenous inoculation is without effect. Infected animals develop a hypersensitivity but no immunity to reinfection.[9]

There is little or no immunity. The chancroid is frequently multiple and is auto-inoculable. A hypersensitivity develops, which is demonstrable as a reaction to the intradermal inoculation of killed bacilli and which persists for many years. The disease may be successfully treated with the common drugs with the exception of penicillin; chlortetracycline is regarded by many as the drug of choice, and streptomycin has given excellent results.

Hemophilus Vaginalis. Another hemophilic microorganism has been described as occurring in association with, and possibly etiological relation to, human vaginitis.[16] Like the other members of the group, *H. vaginalis* is a small, nonencapsulated, nonmotile gram-negative bacillus. It is somewhat more exacting in its nutritive requirements than the other Hemophilus species, is relatively inactive biochemically, and the delicate growth is facilitated by incubation in increased carbon dioxide tension.[13, 39] Penicillin may be incorporated into the medium (10μ/ml.) for primary isolation from contaminated specimens. It is reported to be clearly distinguishable serologically, by growth requirements, and cultural characteristics.[12]

REFERENCES

1. Alexander, H. E., Leidy, G., and MacPherson, C. F. C.: Production of types A, B, C, D, E and F *H. influenzae* antibody for diagnostic and therapeutic purposes. J. Immunol., 1946, **54:**207–211.
2. Andersen, E. K.: Serological studies on *H. pertussis, H. parapertussis* and *H. bronchisepticus.* Acta Path. Microbiol. Scand., 1953, **33:**202–224.
3. Armitage, P., and Perry, W. L. M.: British standard for pertussis vaccine: Its use in routine control of commercial vaccines. Brit. Med. J., 1957, **ii:**501–505.

4. Asin, J.: Chancroid. A report of 1,402 cases. Amer. J. Syph., 1952, **36:**483–487.
5. Brøchner-Mortensen, K., Engbaek, H. C., and Schmith, K.: Specific serum treatment for Pfeiffer meningitis. Acta Med. Scand., 1948, **31:**129–145.
6. Buddingh, G. J.: Experimental combined viral and bacterial infection (influenza C and *Hemophilus influenzae* type B) in embryonated eggs. J. Exptl. Med., 1956, **104:**947–958.
7. Crawford, J. G., and Fishel, C. W.: Growth of *Bordetella pertussis* in tissue culture. J. Bact., 1959, **77:**465–474.
8. Crook, W. G., Clanton, B. R., and Hodes, H. L.: *Hemophilus influenzae* meningitis. Observations on the treatment of 110 cases. Pediatrics, 1949, **4:**643–659.
9. Dienst, R. B.: Virulence and antigenicity of *Hemophilus ducreyi*. Amer. J. Syph., 1948, **32:** 289–291.
10. Dolby, J. M.: The separation of the histamine-sensitizing factor from the protective antigens of *Bordetella pertussis*. Immunol., 1958, **1:** 328–337.
11. Donald, W. D., and Coker, J. W.: The role of *Hemophilus influenzae* in respiratory infections of premature infants. J. Dis. Children, 1957, **94:**272–276.
12. Dukes, C. D., and Gardner, H. L.: Identification of *Haemophilus vaginalis*. J. Bact., 1961, **81:** 277–283.
13. Edmunds, P. N.: The growth requirements of *Haemophilus vaginalis*. J. Path. Bact., 1960, **80:**325–335.
14. Evans, D. G., and Perkins, F. T.: The ability of pertussis vaccine to produce in mice specific immunity of a type not associated with antibody production. Brit. J. Exptl. Path., 1954, **35:**322–330.
15. Evans, D. G., and Perkins, F. T.: The production of both interference and antibody immunity by pertussis infection in mice. Brit. J. Exptl. Path., 1955, **36:**391–401.
16. Gardner, H. L., and Dukes, C. D.: *Haemophilus vaginalis* vaginitis. A newly defined specific infection previously classified "nonspecific" vaginitis. Amer. J. Obstet. Gynecol., 1955, **69:** 962–976.
17. Gilder, H., and Granick, S.: Studies on the Hemophilus group of organisms. Quantitative aspects of growth on various porphin compounds. J. Gen. Physiol., 1947, **31:**103–117.
18. Glynn, A. A.: Antibodies to *Haemophilus influenzae* in chronic bronchitis. Brit. Med. J., 1959, **ii:**911–914.
19. Gordon, J. E., and Hood, R. I.: Whooping cough and its epidemiological anomalies. Amer. J. Med. Sci., 1951, **222:**333–361.
20. Granick, S., and Gilder, H.: The structure, function and inhibitory action of porphyrins. Science, 1945, **101:**540.
21. Granick, S., and Gilder, H.: The porphyrin requirements of *Haemophilus influenzae* and some functions of the vinyl and propionic acid side chains of heme. J. Gen. Physiol., 1946, **30:**1–13.
22. Hazen, L. N., et al.: Antibiotic treatment of pertussis. Comparison of penicillin, Aureomycin, chloramphenicol and Terramycin in 150 cases. J. Pediatrics, 1951, **39:**1–15.
23. Kaufman, S., and Bruyn, H. B.: Pertussis. A clinical study. J. Dis. Children, 1960, **99:**417–422.
24. Kendrick, P. L., Nadolski, E. B., Eldering, G., and Baker, J.: Antigenic relationships of *Haemophilus pertussis*, the parapertussis bacillus, and *Brucella bronchiseptica* as shown by cross protection tests in mice. J. Bact., 1953, **66:**166–169.
25. Kind, L. S.: The altered reactivity of mice after inoculation with *Bordetella pertussis* vaccine. Bact. Rev., 1958, **22:**173–182.
26. Koch, R., and Carson, M. J.: Management of *Hemophilus influenzae* type b meningitis. Analysis of 128 cases. J. Pediatrics, 1955, **46:** 18–29.
27. Lautrop, H.: Laboratory diagnosis of whooping-cough or Bordetella infections. Bull. W. H. O., 1960, **23:**15–31.
28. MacPherson, C. F. C.: A method of typing *Hemophilus influenzae* by the precipitin reaction. Canadian J. Res., Sec. E, 1948, **26:** 197–199.
29. MacPherson, C. F. C., Heidelberger, M., Alexander, H. E., and Leidy, G.: The specific polysaccharides of types a, b, c, d and f *Hemophilus influenzae*. J. Immunol., 1946, **52:**207–219.
30. McCrumb, F. R., Jr., et al.: Treatment of *Hemophilus influenzae* meningitis with chloramphenicol and other antibiotics. J. Amer. Med. Assn., 1951, **145:**469–474.
31. Miller, J. J., Leach, C. W., Saito, T. M., and Humber, J. B.: Comparison of the nasopharyngeal swab and the cough plate in the diagnosis of whooping cough and *Hemophilus pertussis* carriers. Amer. J. Pub. Health, 1943, **33:**839–843.
32. Mishulow, L., Sharpe, L. S., and Cohen, L. L.: Beef-heart charcoal agar for the preparation of pertussis vaccines. Amer. J. Pub. Health, 1953, **43:**1466–1472.
33. Olitzki, A. L., and Sulitzeanu, A.: Antigenic structure of *Haemophilus aegyptius* and *Haemophilus influenzae* demonstrated by the gel precipitation technique. J. Bact., 1959, **77:**264–269.
34. Pittman, M.: Sensitivity of mice to histamine during respiratory infection by *Haemophilus pertussis*. Proc. Soc. Exptl. Biol. Med., 1951, **77:**70–74.
35. Pittman, M., and Davis, D. J.: Identification of the Koch-Weeks bacillus (*Hemophilus aegyptius*). J. Bact., 1950, **59:**413–426.
36. Powell, H. M., Culbertson, C. G., and Ensminger, P. W.: Charcoal agar culture medium for preparing *Hemophilus pertussis* vaccine. Pub. Health Repts., 1951, **66:**346–348.
37. Report: Treatment of whooping-cough with antibiotics. Lancet, 1953, **i:**1109–1112.
38. Reymann, F.: A study of the growth conditions of *Haemophilus ducreyi*. Acta Path. Microbiol. Scand., 1947, **24:**208–212.
39. Ritzerfeld, W., and Kümmel, J.: Untersuchungen zur bakteriologischen Diagnostik von *"Haemophilus vaginalis."* Zent. f. Bakt., I Abt. Orig., 1960, **180:**334–342.
40. Robbins, K. C., and Pillemer, L.: Separation of pertussal toxin. Proc. Soc. Exptl. Biol. Med., 1950, **74:**75–78.
41. Shaffer, M. F., and Shaffer, L. S.: Infectivity of *Hemophilus pertussis* for the chick embryo. Proc. Soc. Exptl. Biol. Med., 1946, **62:**244–245.
42. Standfast, A. F. B.: The virulence of *Haemophilus pertussis* for mice by the intranasal route. J. Gen. Microbiol., 1951, **5:**250–266.

43. Ström, J.: Is universal vaccination against pertussis always justified? Brit. Med. J., 1960: **ii:**1184–1186.
44. Thomson, D.: Whooping-cough—a review. Monthly Bull. Ministry of Health Pub. Health Lab. Service, 1953, **12:**92–102.
45. Toulant, P., Larmande, A., and Toulant, M.: Les conjonctivités purulentes endémo-épidémiques des pays chauds. Bull. Soc. Path. Exot., 1951, **44:**549–553.
46. Ungar, J., et al.: The cultivation of *Haemophilus pertussis* in a partially defined liquid medium. J. Gen. Microbiol., 1950, **4:**345–359.
47. Ungar, J., Muggleton, P. W., and Stevens, W. K.: The type specificity of *Haemophilus pertussis*. J. Hyg., 1954, **52:**475–485.
48. Verwey, W. F., and Thiele, E. H.: Studies on the antigenicity of toxic extracts of *Hemophilus pertussis*. J. Immunol., 1949, **61:**27–33.
49. Verwey, W. F., Thiele, E. H., Sage, D. N., and Schuhardt, L. F.: A simplified liquid culture medium for the growth of *Hemophilus pertussis*. J. Bact., 1949, **58:**127–134.
50. Waterworth, P. M.: The stimulation and inhibition of growth of *Haemophilus influenzae* on media containing blood. Brit. J. Exptl. Path., 1955, **36:**186–194.
51. Williamson, G. M., and Zinnemann, K.: The occurrence of two distinct capsular antigens in *H. influenzae* type e strains. J. Path. Bact., 1951, **63:**695–698.
52. Zamenhof, S., et al.: Polyribophosphate, the type-specific substance of *Hemophilus influenzae*, type b. J. Biol. Chem., 1953, **203:**695–704.

Chapter 27

PSEUDOMONAS; LACTOBACILLUS; LISTERIA; ERYSIPELOTHRIX; BACTEROIDES; STREPTOBACILLUS; BARTONELLA; PLEUROPNEUMONIA GROUP; DONOVANIA

PSEUDOMONAS AERUGINOSA (PYOCYANEA)

The genus Pseudomonas includes some thirty species which are found, for the most part, in water, soil, and wherever organic matter is decomposing. The fluorescent bacteria are members of this genus, *Pseudomonas fluorescens* being the gelatin-liquefying form and *Ps. nonliquefaciens* the nonliquefying form. The elaboration of blue pigment by *Ps. syncyanea* results in "blue milk." One species, *Ps. septica,* is the cause of a disease of caterpillars, another, *Ps. reptilovorus,* produces disease in certain reptiles.

The best known species of Pseudomonas, however, and the only one that is pathogenic for man, is *Ps. aeruginosa,* which is also commonly known as *Ps. pyocyaneus.* The blue or blue-green stains that sometimes appear upon surgical dressings long ago attracted attention, and even before the cause of the phenomenon had been discovered Fordos studied the pigment in 1860. Gessard found, in 1882, that the pigment was the product of a specific microorganism, *Ps. aeruginosa,* which he isolated in pure culture.

Morphology and Staining. The cells of *Ps. aeruginosa* vary considerably in size and proportion but appear usually as small, slender rods, 1.5 to 3 μ long and 0.5 μ broad, frequently united in pairs and short chains. There are one to three polar flagella, and the bacterium is actively motile. Neither capsules nor spores are formed. The colonies are large and spreading, edges are irregular, and the consistency butyrous. These bacilli stain readily with the usual aniline dyes and are gram-negative.

Physiology. *Ps. aeruginosa* grows readily on all the ordinary culture mediums and most rapidly at a temperature of 30° to 37° C. Aerobic conditions are re-

quired, although there is some growth under anaerobic conditions. Gelatin is rapidly liquefied, hydrogen sulfide is produced, and indol is not formed (false positives are sometimes given with Ehrlich's reagent, but not with Kovacs' reagent). The fermentative abilities of this bacterium are limited; acid is produced from dextrose, but most other carbohydrates are not attacked. An alkaline reaction is produced in litmus milk; a soft coagulum is formed, followed by rapid peptonization and reduction of the indicator.

One of the most distinctive characteristics of *Ps. aeruginosa* is its production of a bluish green, soluble pigment which does not color the colonies or other masses of growth but instead diffuses into the medium. There are actually two pigments formed. The one, *pyocyanin,* is a deep blue in color and can be extracted from aqueous solution by chloroform, and the other, a yellowish green flourescent pigment, is soluble in water but not in chloroform. They are both oxidation products of colorless precursors. Pyocyanin is formed only by *Ps. aeruginosa* but the fluorescent pigment is formed by several other species of Pseudomonas.[25] These pigments may occur separately or together. Pyocyanin is of interest in that it is a phenazine dye.

The serologic character of Pseudomonas, especially *Ps. aeruginosa,* was open to question for many years.[34] It is now established that there is antigenic diversity within the species with respect to both H and O antigens, allowing the differentiation of 10 O groups, which in combination with the 10 H antigens, has allowed the separation of 29 agglutinative serotypes.[61] There appears to be no species-specific antigen, but the O antigens of *Ps. aeruginosa* seem not to occur in other Pseudomonas species.

Pathogenicity. For some time after its discovery *Ps. aeruginosa* was generally regarded as a harmless saprophyte, or at the most as a microorganism of slight pathogenic power. It subsequently became clear that this bacterium is causally associated with a great variety of suppurative and other affections in man. Apart from the many doubtful cases in which *Ps. aeruginosa* is found mixed with streptococci, staphylcocci, and other microorganisms, where its share in inciting pathological processes is problematical, numerous instances are on record in which little or no question exists as to its etiological role. It is found in pure culture in abscesses in different parts of the body, especially in the middle ear.[54] Cases of endocarditis,[35] pneumonia and meningitis,[41] though rare, also occur in which *Ps. aeruginosa* appears to be the sole responsible microorganism. A generalized and fatal form of pyocyanic infection occurs, and the bacillus has been found in the blood during life.[7] *Ps. aeruginosa* has been found constantly present in the intestinal discharges of patients

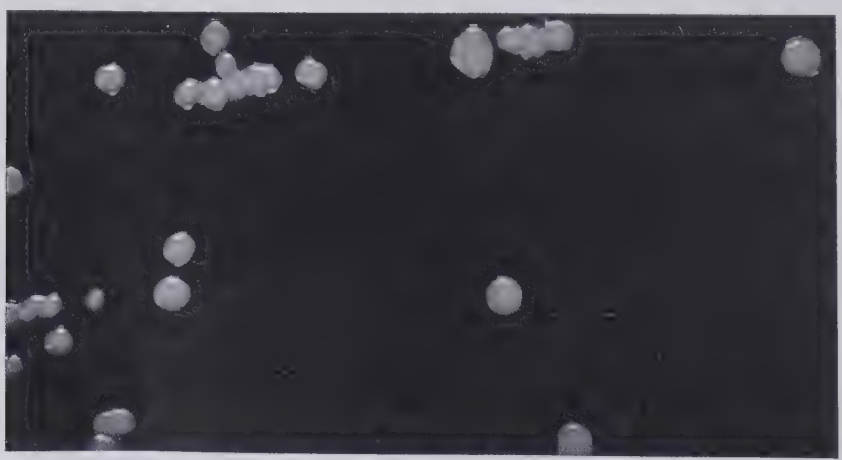


Figure 142. Colonies of *Pseudomonas fluorescens* on nutrient agar; 24-hour culture; × 3.

during a dysentery-like epidemic, and the same microorganism was also present in abundance in drinking water which seemed, on epidemiological grounds, to be implicated in the outbreak.[29]

Infections with *Ps. aeruginosa* and other Pseudomonas species are difficult to treat because they do not respond well or uniformly to antibacterial drugs. In general, polymyxin is the most consistently active, streptomycin and the tetracyclines are less active and give variable results, and penicillin, erythromycin, and other antibiotics affecting the gram-positive bacteria have substantially no therapeutic effect.[2]

The intraperitoneal injection of 0.25 ml. of culture of a virulent strain will kill a guinea pig with acute symptoms in 24 hours. Smaller amounts are also fatal but less rapidly. Subcutaneous inoculation produces a marked local reaction. The symptom complex presents nothing especially characteristic. Rabbits are not so susceptible as guinea pigs; mice and pigeons are less susceptible than rabbits. Immunity can be produced by small, nonlethal doses.

Pseudomonas appears to occupy something of a unique position in that it is not only potentially pathogenic for man and other animals, but is pathogenic for plants also. The plant pathogen, *Phytomonas polycolor,* seems to be identical with *Ps. aeruginosa.*[12]

LACTOBACILLUS

Microorganisms comprising this somewhat loosely assembled genus produce considerable amounts of lactic acid from the simpler carbohydrates and concomitantly withstand a degree of acidity usually fatal to nonsporulating bacteria. This acid-tolerant attribute of these bacteria, which are termed aciduric, is extremely useful in the isolation of cultures as well as in distinguishing the group.

Morphologically some of the lactobacilli are long, slender rods, while others are somewhat similar to the colon bacillus, but, unlike it, they are all gram-positive. Almost all are nonmotile, but exceptions have been reported. Many cultures exhibit a typical diplobacillary form, often kidney-shaped. Old cultures frequently show considerable pleomorphism.

Physiology. The lactobacilli are micro-aerophilic or anaerobic on primary isolation, but after continued cultivation some strains will grow in the presence of air. Their nutritional requirements are complex, and most strains cannot be cultivated on the usual nutrient or infusion mediums unless these are enriched by the addition of glucose or whey. Individual requirements for amino acids vary from two to fifteen, and pyridoxine, thiamin, riboflavin, biotin, folic acid, and nicotinic acid are required in general, individual requirements differing. Such varied nutritional requirements have valuable practical application in microbiological assay procedures for vitamins and certain amino acids for they are more sensitive than available chemical methods. Within an appropriate concentration range, there is a definite, even linear, relation between the concentration of vitamin in a vitamin-free but otherwise nutritionally adequate culture medium and the amount of growth or acid produced.

Some lactobacilli are a part of the normal intestinal flora and may predominate in infants and others having a high intake of sugars, especially lactose. At one time it was supposed that a lactobacillus intestinal flora is preferrable to a coliform proteolytic flora because it tended to inhibit degenerative changes with increased vitality in the higher age groups, and that such a flora could be established by consumption of fermented milks, acidophilus and bulgaricus milks. The composition of the intestinal flora may be altered in this way, and also by the consumption of equal amounts of sweet milk,

but the change is temporary, and in any case there is no evidence that such an intestinal flora in itself favors general health.

While rare instances of association of lactobacilli with pathological conditions, such as endocarditis and febrile disease, have been noted, these bacteria are essentially nonpathogenic except insofar as they may be associated with dental caries (see below). They are primarily of interest in the dairy and fermentation industries where they are of considerable importance.

Classification. The classification of lactobacilli has been based on the source from which they were isolated. Such a basis is unsatisfactory except possibly in the case of certain obligate parasitic bacteria having highly pronounced host specificities. No other means of differentiation and characterization has been completely satisfactory and, although physiological properties vary widely from strain to strain, at the present time species of lactobacilli are separated on a physiological basis, using optimum growth temperatures, anaerobiasis, and certain sugar fermentations.

The lactobacilli fall into two groups on the basis of the products of sugar fermentation. The homofermentative group, which is the largest, converts the fermented sugar almost entirely to lactic acid, while the heterofermentative group is made up of the forms which produce considerable amounts of other products of fermentation, including carbon dioxide, ethanol, and acetic acid. According to the Bergey classification, these two groups are divided into species in the following way:

Homofermentative lactobacilli:
- (A) having an optimum temperature of 37° C. to 60° C. or higher
  - (1) produce acid from lactose
    - (a) having an optimum temperature of 37° C. to 45° C.
      - (i) produce *l*-lactic acid
        Lactobacillus caucasicus
        Lactobacillus lactis
      - (ii) produce *dl*- or *d*-lactic acid micro-aerophilic
        Lactobacillus helveticus
        Lactobacillus acidophilus
      - (iii) anaerobic in freshly isolated cultures
        Lactobacillus bifidus
    - (b) having an optimum temperature of 45° C. to 62° C.
      Lactobacillus bulgaricus
      Lactobacillus thermophilus
  - (2) do not produce acid from lactose
    Lactobacillus delbrueckii
- (B) having an optimum temperature between 28° C. and 32° C.
  - (1) produce optically active lactic acid
    - (a) produce *d*-lactic acid
      Lactobacillus casei
    - (b) produce *l*-lactic acid
      Lactobacillus leichmannii
  - (2) produce optically inactive lactic acid
    Lactobacillus plantarum

Heterofermentative lactobacilli:
- (A) having an optimum temperature of 28° C. to 32° C.

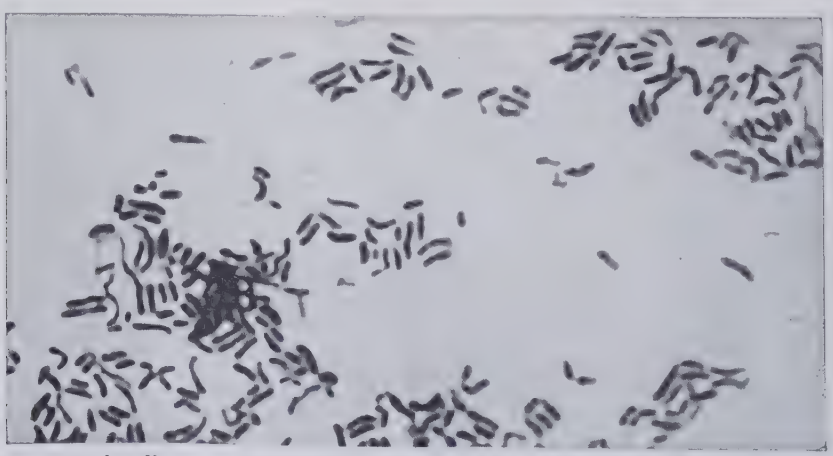


Figure 143. *Lactobacillus* sp. isolated from the mouth. Morphologically identical with *L. acidophilus*. Note the diplobacillary form and palisade arrangement of the cells; × 2500. (Harrison.)

(1) ferment raffinose, sucrose, and lactose
Lactobacillus pastorianus
Lactobacillus buchneri
(2) do not ferment raffinose, and often not sucrose or lactose
Lactobacillus brevis
(B) having an optimum temperature of 35° C. to 40° C. or higher
Lactobacillus fermenti

A number of studies of the serological relationships among the lactobacilli have been carried out, using polysaccharides extracted from the bacteria as antigens, and the conventional agglutinin-agglutinin absorption methods. In the first instance, four serological types were separated but appeared to be somewhat unstable, changes in antigenicity being associated with changes in fermentation reactions.[21, 23, 24] While the study of agglutinogens is complicated by the marked tendency of lactobacilli to spontaneous agglutination in salt solution, ten different agglutinogens are demonstrable among the homofermentative lactobacilli, together with certain minor antigens shared with Leuconostoc species.[38, 64, 65]

Of the lactobacillus species distinguished by physiological reactions, only three need be considered here.

Lactobacillus Acidophilus. First cultivated by Moro in 1900 from the feces of infants, this organism has been isolated from the intestine of nearly all the mammalia, many other vertebrates, and some of the invertebrates. Its number increases in the intestine when the carbohydrate content of the diet is increased and may become predominant when a milk diet is ingested. These bacilli, fairly large and of variable length, are arranged singly, in pairs frequently slightly bent at the juncture, and in palisades. Long chains, filamentous forms, and club shapes are not uncommon. Young cultures stain uniformly gram-positive; old cultures often show beading or bipolar staining and may be easily destained. Colonies, usually small, may vary in shape between a smooth, rounded, opaque form and a flattened, translucent, irregular form often having a ground-glass appearance. Fermentation reactions are variable, but most strains produce acid, no gas, from glucose, lactose, maltose, and sucrose and coagulate milk within 48 hours. Döderlein's bacillus (1892), a common constituent of the flora of the vagina and believed to aid in the natural defenses against infection by contributing to the acidity of the vaginal secretions, is said to be identical with *L. acidophilus.*

Lactobacillus Bifidus. Apparently closely related to *L. acidophilus* and often difficult to distinguish from it, *L. bifidus* is a thinner rod with ends somewhat more tapering and usually bifurcated on isolation. It was isolated from feces of breast-fed infants by Tissier in 1900. Although common in the intestine of breast-fed infants, sometimes comprising over 90 per cent of the total intestinal flora, it is less conspicuous in the intestinal contents of bottle-fed babies. It is sometimes found also in the feces of adult animals, including man. Like *L. acidophilus* it produces acid, mainly lactic, from a number of sugars but also ferments inulin. It is anaerobic on primary isolation, and some strains never grow well under aerobic conditions. Growth is enhanced by cystine. Partly because of its anaerobic requirements it has been classified with the Bacteroides.

Lactobacillus Bulgaricus. This name was assigned to an organism isolated by Grigoroff in 1905 from Bulgarian fermented milk. It gained prominence through the work of Metchnikoff, who believed that intestinal putrefaction could be restrained by drinking milk fermented by this organism as noted above.

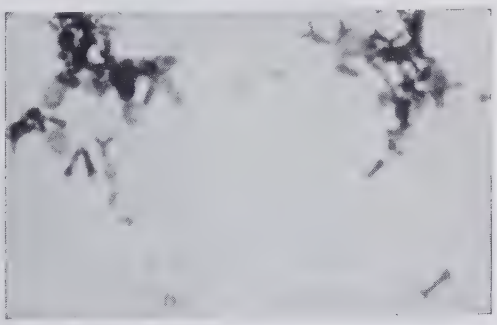


Figure 144. *Lactobacillus bifidus.* Note the Y-shaped forms. (Dack.)

When it was later shown that *L. bulgaricus* does not become implanted in the intestine, its use in experimental therapeusis was dropped in favor of *L. acidophilus*. More difficult to cultivate than *L. acidophilus*, slightly larger, and somewhat different in sugar fermentations, it is nevertheless closely related. It has been reported that *L. bulgaricus* rarely grows at 15° C., dies out on repeated culture in a lactose-peptone-yeast extract broth, is unable to grow in mediums containing 2.5 per cent sodium chloride and fails to grow in broth at pH 7.8, while *L. acidophilus* will grow under all these conditions. The Boas-Oppler bacillus, first seen in 1895 in the gastric juice of patients suffering from stomach carcinoma, is a member of this group, similar to if not identical with *L. bulgaricus*.

DENTAL CARIES AND PERIODONTAL DISEASE

Decalcification of the tooth, which is a prominent part of dental caries, may be brought about by organic acids of microbial origin. Since the lactobacilli are a part of the normal flora of the mouth, and both acid-forming and aciduric, it has been suspected that they may be etiologically related to the carious process.

Microorganisms gain access to the mouth at birth, and a normal flora, which consists of those forms which are able to persist, is established by the time deciduous dentition has been formed. While it is subject to variation associated with diet or disease, the microorganisms comprising the normal flora, living on the oral mucosa, in and about the gum margins and on the teeth, are characteristic of the oral cavity.[22, 28] The flora includes, in addition to lactobacilli, filamentous forms both branching (actinomyces) and nonbranching (leptotrichia), α-hemolytic streptococci or enterococci, miscellaneous gram-positive cocci, Neisseria, diphtheroid bacilli, fusiform bacilli and other nonsporulating anaerobic forms, pleuropneumonia-like organisms, spirochetes, yeasts, and monilia. Not all of these are readily cultivable, and the numbers and kinds of microorganisms cultured from saliva or tooth plaques differ from those found in direct smears, and it is probable

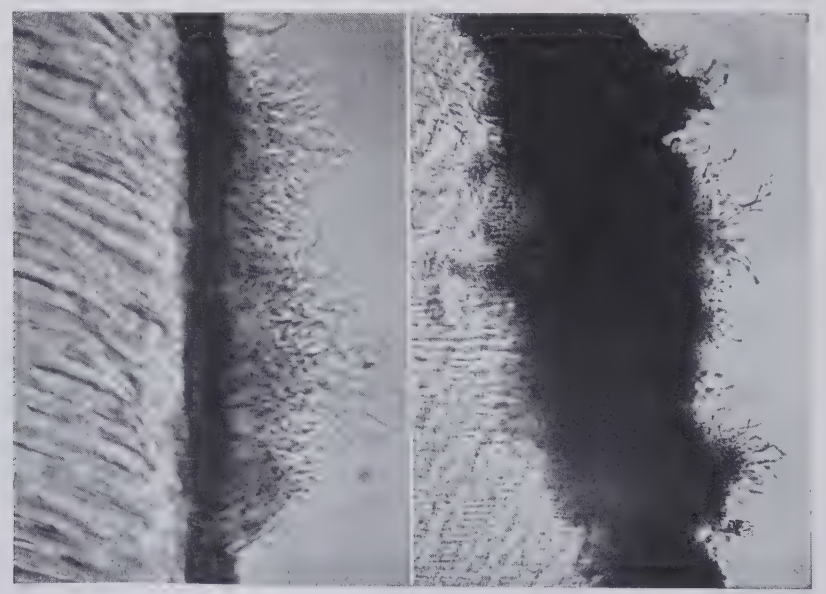


Figure 145. *Left,* ground section of human tooth enamel showing an unstained bacterial plaque growing on the surface. Note the enamel rods and filamentous bacteria; × 1200. (Blayney.) *Right,* ground section through an early carious lesion in human tooth enamel stained with gentian violet. Note the intimate contact of the bacterial plaque with the eroding surface; × 500. (Blayney.)

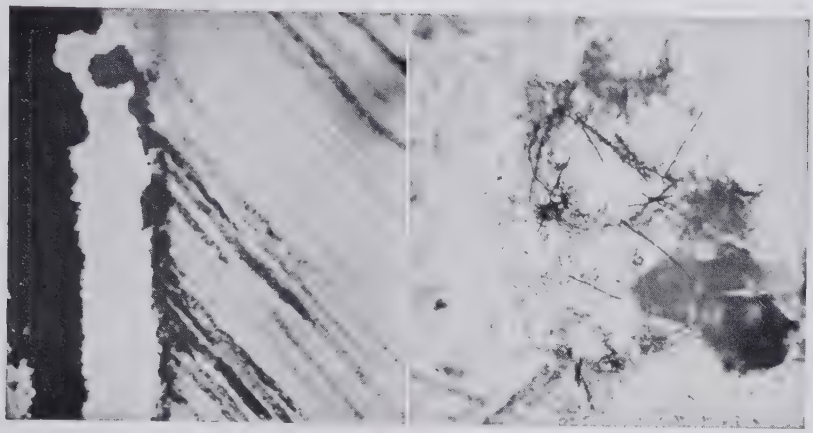


Figure 146. *Left*, ground section of carious human dentin showing bacteria in the dental tubules; × 1000. (Blayney.) *Right*, smear material from a fragment of a bacterial plaque like that illustrated in Figure 145, *right*. Note the bacillary forms which have been teased out of the filamentous masses: × 1000. (Blayney.)

that any single method of assay provides at best a distorted reflection of the microbial flora present.

The composition of the flora is affected by the nature of the diet, including dietary deficiencies, and by other factors such as antibiotic therapy which often results in a predominantly coliform flora. It is also significantly altered when certain pathological conditions are present in the mouth, and it is assumed that such alterations may reflect any part played, particularly by those forms which increase in numbers, in the pathological process.

In general, gingivitis and periodontal disease are associated with a predominantly proteolytic flora, while dental caries is often accompanied by a proportionate increase in fermentative types of microorganisms, especially lactobacilli. In either case, the flora remains highly complex, and inferences of etiological relationship may be made only tentatively.

In the first instance, involvement of the gum margins may range from mild involvement of the buccal and lingual margins and interdental papillae to the acute or chronic ulceromembranous gingivitis known as Vincent's disease, and may extend into the periodontal membrane to produce suppurative periodontitis or pyorrhea. A variety of microorganisms are found in such lesions. The classic microscopic picture of spirochetes and fusiform bacilli diagnostic of Vincent's disease is incomplete, and cocci, filamentous forms, and gram-negative bacilli are also present in the masses of bacteria growing along the gingival margin. Rosebury,[52] for example, has found that such fusospirochetal exudates contain 21.1 per cent spirochetes, 2.8 per cent fusiform bacilli, 66.7 per cent streptococci, 4.1 per cent gram-negative nonmotile bacilli, and 3 per cent gram-positive bacilli. Since all of these microorganisms are recovered from normal gingivae, though in smaller numbers, it becomes difficult to differentiate between an etiological role and multiplication in the substrate provided by the necrotic tissue. To complicate matters further, there is some evidence of synergism, *i.e.*, that pure cultures are not pathogenic, but a mixture of a spirochete (Treponema), a fusiform bacillus, a vibrio, and an anaerobic streptococcus produces a characteristic, transmissible infection in experimental animals.[52]

The difficulties are much the same in attempting to assign an etiological role to one or another kind of bacteria, including lactobacilli, in the development of the carious process. It has been generally, though not invariably, found that the number of lactobacilli present in the saliva increases during active caries and that both the development of the carious process and the increase in numbers of

lactobacilli are arrested by sharp restriction of sugars in the diet. The carious lesion is commonly initiated beneath a tooth plaque, a felt-like mass on the surface of the tooth made up of filamentous microorganisms in which various kinds of bacteria are entrapped. The predominant forms are α-hemolytic streptococci, gram-positive diplococci, diphtheroid bacilli, and anaerobic fusiform bacilli, together with smaller numbers of lactobacilli, Neisseria, and other gram-negative cocci, etc.

There seems to be no doubt that bacteria are intimately associated with the development of the carious process, for experimental caries does not occur in germ-free animals.[39] Few are demonstrable along the advancing edge of the initial lesion, but sections show that Tome's fibrils are invaded by bacteria, followed by decalcification of the dentinal tubular contents and subsequently of the dentinal matrix, and the decalcified tubules become filled with bacteria. Two kinds of biochemical processes are involved; decalcification, presumably by organic acids of microbial origin; and the proteolytic activity of microorganisms. In the first instance, the rapid production of relatively large amounts of lactic acid by lactobacilli makes these bacteria likely candidates as etiological agents, but it is also true that enterococci produce sufficient acid, lactic acid for the most part, for decalcification. So far as proteolytic activity is concerned, a variety of microorganisms of the normal flora are able to bring about the observed changes, but in general bacterial proteases are tryptic rather than peptic in nature and active in alkaline solution so it is unlikely that proteolysis and decalcification occur simultaneously.

In summary, in both periodontal disease and caries, the microorganisms found in association with such processes are those of the normal flora of the mouth, but in altered proportions. It is probable that some of those found in increased numbers are responsible, either alone or in combination, for the observed pathological changes, while others increase in numbers because of the favorable substrate for growth which the lesion provides and, although adventitious, may contribute to the process. It seems unlikely that either process is initiated by microorganisms, but rather that it is related originally to the resistance of the host, especially as affected by diet, but, given favorable conditions, the microorganisms play an essential part in the development of the lesion. The etiological relation of the lactobacilli to dental caries thus becomes blurred, but this does not invalidate the hypothesis that these bacteria play a significant, and possibly essential, part in the development of dental caries.

RELATED BACTERIA

Microbacterium. Forms which are closely related to the lactobacilli and produce lactic acid without gas in carbohydrate fermentation, but which are aerobic, are grouped in the genus Microbacterium and formally classified with Listeria and Erysipelothrix as Corynebacteriaceae. Two species are recognized, *Microbacterium lacticum* and *M. flavum*. The first occurs in the intestinal tract, and the second predominantly in dairy products. Their relatively high heat resistance is of significance in the latter.

Propionic Acid Bacteria. A group of bacteria closely related to the lactobacilli, but characterized by the production of propionic acid and acetic acid in the fermentation of carbohydrates and polyhydric alcohols, is known as the propionic acid bacteria. The original single species, *Bacterium acidi propionici,* has been split into a number of species on a physiological basis under the generic name Propionibacterium. Of the eleven species recognized by Bergey all but one are anaerobic or micro-aerophilic.

They are nonmotile, non-spore-forming, gram-positive bacilli somewhat resembling the diphtheroids in microscopic morphology in that they contain metachromatic granules, and the cells are often club-shaped and show branching.

Growth is relatively slow, and their nutritional requirements are complex in that yeast extract mediums supplemented with lactate or monosaccharides are required to support growth. These bacteria are of some industrial importance and have been widely used in studies on the mechanisms of bacterial fermentation of sugars. They seem to be completely nonpathogenic.

LISTERIA MONOCYTOGENES[55]

The microorganism now known as *Listeria monocytogenes* has undoubtedly been isolated from diseased lower animals and man prior to 1926, but without sufficiently precise characterization to allow its identification. In that year it was described by Murray, Webb and Swann as the etiologic agent of an epizootic among laboratory rabbits and guinea pigs which was characterized in part by a monocytosis, and it was named by them *Bacterium monocytogenes*. What proved to be the same microorganism was isolated the following year by Pirie from the gerbil (*Tatera lobengulae*) in South Africa, and named by him *Listerella hepatolytica* because of the lesions produced in the liver in experimentally infected animals. When the identity of these isolates was established, the microorganism was given the name *Listerella monocytogenes*. Following the objection of systematists that the generic name Listerella had been pre-empted, the name was changed to *Listeria monocytogenes* by which it continues to be known despite further objections by systematists that this generic name had also been preempted. It has been isolated since but not recognized owing to insufficiently critical characterization, and the names *Corynebacterium infantisepticum* and *Corynebacterium parvulum* occur in the literature.

Morphology and Staining. This microorganism is a nonsporulating, nonencapsulated rod with slightly pointed ends. In very young cultures it is found in bacillary form, but later it is predominantly coccoid, 0.5 $\mu \times 1$ to 2 μ, the form in which it is usually observed. In old cultures or cultures of the rough form, filaments 6 to 20 μ in length occur. The arrangement of the cells is not characteristic; they may be found singly, in pairs sometimes at an acute angle to one another, and in short chains.

In young cultures the bacilli are invariably gram-positive, but as the culture ages the Gram reaction becomes irregular. On partial decolorization as observed in older cultures, the center por-

Figure 147. *Listeria monocytogenes.* Smear from a pure culture. Fuchsin; × 1050.

tion of the cell is decolorized first to suggest bipolar staining, but metachromatic granules and the banded appearance characteristic of the Corynebacteria does not occur. Some of the conventional simple stains, *e.g.*, methylene blue, are not satisfactory, and the Gram stain or Giemsa should be used, especially for tissue sections.

These bacteria are motile, a characteristic which, among others, distinguishes them from Erysipelothrix (with which they are classified by British workers as *Erysipelothrix monocytogenes*), and motility is demonstrable by swarming in soft agar culture at 20° C. as well as by direct observation. When grown at room temperature there are 4 or less peritrichous flagella, but when cultured at 37° C. usually only a single polar flagellum is demonstrable.

Growth is diffuse in liquid media, except in the case of rough strains, and on solid media, *e.g.*, tryptose, blood or serum agar, minute opaque colonies appear in 24–48 hours, becoming larger with raised edges on continued incubation. There is a very small zone of β-hemolysis; occasional strains show much greater hemolytic activity on sheep blood agar which is correlated with lecithinase activity of the strain. Two main types of variants are found, the one more translucent and less hemolytic which is agglutinable in saline but retains antigenicity, and the other typically rough.

Physiology. Cultural requirements are not exacting but these bacteria are best grown, especially on primary isolation, on relatively rich mediums and under increased CO_2 tension. Growth occurs at neutral or slightly alkaline reactions, and these bacteria are unusual in their ability to grow well at pH's as high as 9.6 and in the presence of 10 per cent, or even more, NaCl. Growth is relatively slow in liquid mediums in the absence of fermentable carbohydrate, and may be delayed for 1 to 2 weeks or not occur at all when the inoculum is small; this may account for some failures to isolate Listeria from pathological material. When fermentable carbohydrate, *e.g.*, glucose, is included in the medium growth is much more rapid but, as the pH falls to 5.6 or less, the bacteria die out in 2 to 3 days. Enrichment occurs when the specimen is allowed to stand for 1 to 3 months in the refrigerator, owing presumably to the ability of these bacteria to grow slowly at such low temperatures, and subsequent subculture may be successful when immediate culture was not.

These bacteria are relatively inactive biochemically, *i.e.*, they are not proteolytic, do not produce indole or H_2S, and fail to hydrolyze urea or reduce nitrates. Dextrose, maltose, levulose, salicin and trehalose are almost invariably fermented but without gas formation; sucrose, lactose, glycerol, xylose and rhamnose are fermented irregularly or only by some strains; and mannitol, dulcitol, arabinose, inositol and raffinose are not fermented. The separation of Listeria into fermentative types has not been useful.

With the exception of the enhanced hemolytic activity associated with lecithinase and the irregular fermentation of some carbohydrates, *List. monocytogenes* is remarkably uniform regardless of source. It is generally agreed, therefore, that there is but one species, and various names arising from presumed host specificity, such as *List. ovis, List. cuniculi, List. bovina, List. gerbilli,* and *List. gallinarum* have no standing.

Antigenic Structure. Earlier studies on the antigenic specificity of *List. monocytogenes* indicated that these microorganisms fall into two general serological types, types 1 and 4 below. One of these appeared to occur in rodents, the other in ruminants, and both in man. More precise studies, based on detailed antigenic analysis, have shown that both heat-labile flagellar and heat-stable somatic antigens are present. These allow differentiation of four main serotypes, designated 1, 2, 3 and 4, and the subdivision of type 4 into subtypes of which type 4a and type 4b appear to be the most important. The antigenic structure of these serotypes is shown in the accompanying table. While *List. mono-*

Antigenic Structure of Listeria Monocytogenes*

SEROTYPE	MAJOR ANTIGENS Somatic O	Flagellar H
1	I, II . . .	A, B
2	I, II . . .	B, D
3	II, IV . . .	A, B
4a	Va, b . . .	A, B . . .
4b	Va, c . . .	A, B, C

* Modified from Seelinger.

cytogenes may be identified and typed by the use of appropriate antiserums, cross-reactions have been found to occur with a number of other bacteria, including strains of *Str. fecalis, Staph. aureus, E. coli,* and various Corynebacterium species. The serodiagnosis of Listeria infection using paired serums from patients is, then, not reliable for rise in titer to Listeria antigen may occur in infections with other microorganisms.

Toxins. The characteristic monocytosis, to as high as 30 per cent of the total white cell count by the fourth day of experimental infection in rabbits, is doubtless due, at least in large part, to the presence of a monocytosis-producing agent in the microorganisms. This is apparently a lipid substance, liberated by mechanical disruption of the cells and extractable with organic solvents such as chloroform, ether, or petroleum ether. The activity is correlated with virulence for it is present in large amount in freshly isolated virulent strains, decreases rapidly coincident with decline in virulence on serial culture on laboratory mediums, and may be restored by animal passage. The partially purified material produces an effect on the hematopoietic system substantially identical with that which occurs in experimental infection, but it does not appear to be otherwise toxic. Whether polysaccharide fractions of the bacilli are toxic as has been reported has not been established. A protein substance, separable from mechanically disrupted cells and precipitable by ammonium sulfate, has been reported to have a virulence-enhancing effect.[42, 43] These bacteria appear to have no endotoxin in the conventional sense.

Pathogenicity.[5, 62] Naturally occurring infection of lower animals and man, or listeriosis, is apparently world-wide in distribution though most of the reported cases have been in this country and Western Europe. There have been several thousand confirmed infections in lower animals, particularly domestic animals, and more than 700 confirmed human cases have been recorded. It is probable that many are missed because the disease cannot be diagnosed with certainty on clinical grounds alone, and because of failure to isolate and/or recognize the microorganism.

In lower animals the disease occurs in both sporadic and epizootic form. It assumes a generalized septicemic form in rodents and in young animals generally, with the development of necrotic foci in the liver. In older animals there is a tendency to involvement of the central nervous system with resulting encephalitis or encephalomyelitis. The infection also has a tendency to localize in the reproductive systems, resulting in abortion in pregnant animals. Among domestic animals the sheep appears to be among the most susceptible and the disease takes a fulminating form with high mortality, at times assuming considerable economic importance; the disease is known as "circling disease" in these animals, but relevant symptoms of central nervous system infection are not invariably present. Cattle are infected also, and swine less often; the horse is infected only occasionally. Epizootics have occurred in colonies of fur-bearing animals such as foxes and chinchillas.

Of the common experimental animals, the mouse and rabbit appear to be among the most susceptible, and fatal infection is readily produced by intravenous or intraperitoneal inoculation. The guinea pig is less susceptible and cannot be fatally infected with regularity. Freshly isolated smooth strains of *List. monocytogenes* appear to be almost invariably highly virulent, and test for virulence is a significant element in its characterization, but, since virulence is lost readily on culture on laboratory mediums, it may appear to differ widely from strain

to strain when culture strains are tested.

Listeriosis in man may be a localized infection, cutaneous or of mucous membranes of varying severity to give upper respiratory symptoms, angina, involvement of regional lymphatics, and at times associated conjunctivitis. Monocytosis is common in this kind of infection. Or it may assume, or progress to, a generalized septicemic form with infection of the reproductive system and transplacental transmission to the fetus, or neonatal infection in pregnant women, or involve the central nervous system with resulting encephalitis. Histologically the lesions are granulomas and focal necroses. Diagnosis cannot be based on symptomatology; the septic form with angina and monocytosis is indistinguishable from infectious mononucleosis of viral or other etiology (Chap. 37), the encephalitis, in which monocytosis is not a constant feature, does not differ significantly from encephalitis of other bacterial or viral etiology, etc. The case fatality rate appears to be high, perhaps 70 per cent when the central nervous system is involved. At the same time, asymptomatic and abortive infections occur and their relative frequency is not known. Tetracyclines and sulfonamides are considered to be reasonably effective chemotherapeutic agents if given early; penicillin is apparently not consistently effective. The microorganism may be isolated from the blood, or bone marrow by sternal puncture, early in the disease, from purulent material from lymph nodes and cutaneous lesions, from swab specimens from infected mucous membranes, and from spinal fluid when the central nervous system is infected.

Epidemiology. The epidemiology of listeriosis is as yet far from clear. It is considered to be primarily a disease of lower animals, possibly with a reservoir of infection in rodents as suggested by the isolation of the microorganisms from such animals in the wild state. Nevertheless, it has been possible in only relatively few instances of confirmed infection to trace the source of infection. Among domestic animals an association with certain feeds, especially silage, has been noted by many workers, and *List. monocytogenes* has, in fact, been isolated directly from silage.[19] The source of contamination of silage is another matter, and a saprophytic stage in the life history of the microorganism cannot be ruled out. It is probable that human infection is acquired from lower animals through direct or indirect contact. With the exception of transplacental infection of the human fetus, or neonatal infection acquired at birth, the disease seems not to be transmitted from man to man.

ERYSIPELOTHRIX RHUSIOPATHIAE[66]

Microorganisms closely related to the actinomycetes have been found to be the causative agents of swine erysipelas and a type of mouse septicemia; they also infect man, and the disease produced is termed erysipeloid to distinguish it from erysipelas of streptococcal etiology. It was thought for a time that the mouse septicemia organism isolated by Koch, the organism isolated from swine by Pasteur and Thuillier and by Löffler, and that found by Rosenbach in human ervsipeloid were distinct species of a genus to which the name Erysipelothrix has been given, and they were called *Erysipelothrix muriseptica, Ery. rhusiopathiae,* and *Ery. erysipeloides* respectively. The first of these, sometimes called *Bact. murisepticum,* is not to be confused with *Pasteurella muriseptica* which also has the same synonym. Other names that have been used are *Ery. rhusiopathiae suis, Ery. porci, Bacillus erysipelatis suis, Actinomyces rhusiopathiae,* and the swine *rotlauf* bacillus. It is now generally agreed that these organisms are identical,

or at least closely related varieties of the same species, for while their morphology is variable they are immunologically identical and are recognized as a single species, *Ery. rhusiopathiae*. These microorganisms are regarded by some workers as related to *L. monocytogenes*.

Morphology and Physiology.[57] *Ery. rhusiopathiae* occurs in two rather well defined morphological types usually designated smooth and rough though their relationship to the S and R variants of bacterial dissociation is not clear. The smooth type appears as a small, slender, sometimes slightly curved, nonmotile, nonsporulating, gram-positive rod. Long chains of bacilli and filaments, sometimes beaded and showing swollen areas, are present in smears of the rough form. Both stain readily and sometimes irregularly with deeply staining granules. Colonies of the smooth form on solid mediums are round, convex, amorphous, water-clear, and small, perhaps 0.1 mm. in diameter, and broth cultures are uniformly turbid. The rough form produces larger colonies with a granular, curled appearance like that of very small colonies of anthrax bacilli, while growth in liquid mediums is in the form of flocculent, hairlike masses with little or no turbidity. The most characteristic growth is in gelatin stab cultures; beadlike colonies appear along the line of inoculation from which lateral filamentous growth occurs, resembling a test-tube brush.

The organism is micro-aerophilic but will grow under aerobic or anaerobic conditions; growth appears on the usual infusion mediums with 24 hours' incubation at the optimum temperature of 30° C. There is no growth on potato. Fermentation reactions are variable from strain to strain, but most ferment dextrose, lactose, and levulose.[63] Nitrate is reduced and hydrogen sulfide is produced, but indol is not formed, and litmus milk is unchanged or slightly acidified.

Ery. rhusiopathiae is somewhat more than ordinarily resistant to drying and to various preservative processes such as smoking, pickling, and salting and survives for relatively long periods in putrefying meat and in water. It is probably in part because of survival of the organisms in filth that infected areas experience recurrences of the disease year after year.

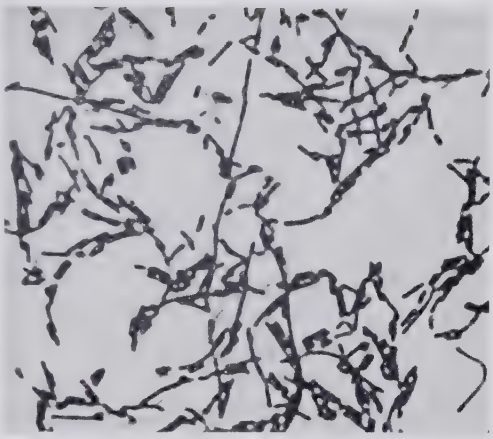


Figure 148. *Erysipelothrix rhusiopathiae.* Pure culture. Note the similarity of this microorganism to the actinomycetes; × 1000. (Kral.)

Pathogenicity. In swine, four clinical types of disease are found. In the acute septicemic form, with lesions in the viscera and internal organs, the case fatality rate is high, perhaps 80 per cent, with death in 3 to 5 days. The urticarial form, known as "diamonds" or "diamond skin disease" because of the occurrence of reddish to purplish rhomboidal blotches on the skin, may occur with or without visceral involvement and is seldom fatal. The chronic form is a vegatative endocarditis with erosion of the mitral valves in particular; death always results eventually. Arthritis may complicate the other clinical types or occur independently; it is usually not fatal, but growth is stunted. The organisms are excreted in large numbers in the feces, and it is generally assumed that natural infection takes place by mouth although experimental feeding has given irregular results. Infection is spread in part by healthy carriers as well as diseased animals, and pork trimmings in garbage probably account for isolated cases. Swine erysipelas is of very great economic importance in Europe, especially in Germany where it is known as *Schweinerotlauf*, and in recent years has been found to be more important in

this country, at least in certain areas, than formerly thought.

Sheep are occasionally infected with *Ery. rhusiopathiae* and develop a polyarthritic form of the disease. The organism is also pathogenic for a variety of birds, and in this country turkeys are most often seriously affected; cyanosis is a prominent clinical feature and evident in the "blue comb." It has also been found in wild rats which should, perhaps, be considered as a reservoir of infection and possibly a source of the human disease.

The disease may be reproduced by the inoculation of swine, though the results are irregular. Of the usual laboratory animals, white mice and pigeons are highly susceptible, dying in 1 to 4 days after subcutaneous inoculation, and are commonly used in diagnosis. The rabbit is not very susceptible, and the guinea pig is quite resistant. In general, the virulence of the microorganism varies widely.

Pathogenicity for man.[30, 48] Human infection with *Ery. rhusiopathiae* is well known. The septicemic type with diffuse erythema is rare in man, and only a very few instances have been reported. The chronic form with endocarditis and polyarthritis is also very rare. The usual type of infection is an erythematous-edematous lesion, the local lesion commonly developing on the fingers or hand from an abrasion where the infection enters. The lesion, although spreading, never extends beyond the wrist. There is some swelling and a marked erythema of the lesion and sometimes local arthritis and regional adenitis. The disease is usually self-limiting and terminates within a month. The organism may be cultivated from an excised piece of skin from the lesion.

Human infection can almost invariably be traced to contact with animals and animal products such as meat, hides, bones, and manure, or to fish and shellfish, and the disease is, therefore, associated with certain occupations. For example, more than half the cases found in the Philadelphia region were in slaughterhouse workers. The disease has been observed in workers in a bone button factory using cattle and hog bones, and it not uncommon in fishermen and fish handlers. It also occurs in persons working in kitchens with raw meat and fish. In this country, contact with live fish and crustacea appears to be the chief source of infection. Hettche has reported finding *Ery. rhusiopathiae* in ten of thirty specimens of sewage at Königsberg in Prussia and in five of fifty-two specimens at Munich; the source apparently was slaughterhouses where infected animals were being killed. Under experimental conditions the organisms not only survived for some days in water, but fish developed a latent infection when fed infected meat; the microorganism could be isolated from most organs, including the kidney, and were excreted in large numbers in the urine, contaminating the water.

Immunity. There is a definite immune response in infected hogs as evidenced by the formation of agglutinins which have diagnostic value. *Ery. rhusiopathiae* is immunologically relatively homogeneous, but serological groups may be distinguished by reciprocal absorption. Antiserum has therapeutic value, and in man is given both intramuscularly and by infiltration about the local lesion. Passive immunization of swine is an effective prophylactic but the immunity lasts no longer than 2 weeks. Animals may be actively immunized with the vaccine developed by Pasteur and Thuillier who attenuated the organism by passing it through rabbits. Vaccine erysipelas occurs with some frequency, however, and the method has now been largely superseded by the simultaneous inoculation of virulent culture and antiserum. Since the vaccine maintains and spreads infection in herds, the Bureau of Animal Industry has permitted only its limited use in this country and only in areas where the disease has become prevalent. Both methods provide adequate protection for 8 to 12 months; periodic reimmunization is required for breeding stock.

NON-SPORE-FORMING ANAEROBIC BACILLI (BACTEROIDES)

There is a large group of non-spore-forming anaerobic bacilli that are usually gram-negative. Normal inhabitants of the upper respiratory tract, genital tract, and colon, where they may outnumber the aerobic flora, the microorganisms are not infrequently associated with ulcerative processes of the mucous membranes and may, under appropriate circumstances, invade the tissues and organs of the body with the production of abscesses, or the blood stream to give rise to septicemia.[3, 13, 26] Generally neglected in routine bacteriological examinations, these bacteria may be present in "sterile pus" from surgically drained abscesses and similar affections in which bacteria are not found by the usual cultural methods. Dack[8] noted their presence in 200 of 5180 specimens from the Department of Surgery of the University of Chicago submitted for routine bacteriological examination, an incidence of about 4 per cent.

The relation of these microorganisms to other bacteria is uncertain. As a group they probably make up several genera and cannot be regarded as species of a single genus except tentatively. They are morphologically heterogeneous, varying from slender rod forms which may be tapered toward the ends, the so-called fusiform bacilli, to filamentous and branching forms characteristic of the higher fungi. The high degree of pleomorphism characteristic of these forms is due in large part to a mode of reproduction in which large round bodies are formed from which daughter cells separate. These usually resemble the bacillary parent cells but sometimes occur as much smaller elements in the so-called L type of variation.[56]

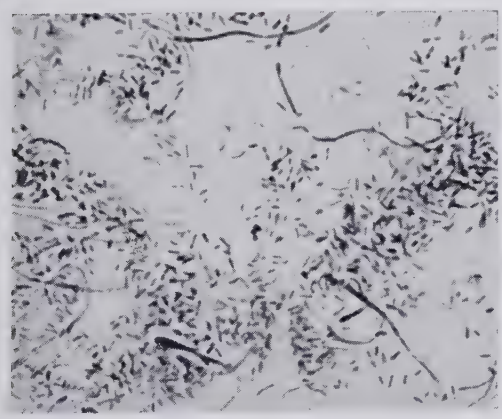


Figure 149. *Bacteroides funduliformis.* The swollen and filamentous forms and poorly staining "ghost cells" are typical of the usual stained smear preparations; × 1000. (Dack.)

Single species have been given a variety of generic names, including Bacillus, Bacterium, Necrobacillus, Bacteroides, Corynebacterium, Streptothrix, and Actinomyces. Descriptions of these bacteria have been compiled and Prevot[47] has suggested a more or less elaborate classification.

The Bergey classification separates these forms between two families and eight genera (exclusive of Streptobacillus) on a morphological basis, *viz.*,

Bacteroidaceae
- Bacteroides (rounded ends)
- Fusobacterium (pointed ends)
  - Dialister (minute, diameter of 150 mμ or less)
  - Sphaerophorus (pleomorphic)

Lactobacillaceae
- Lactobacilleae
  - Eubacterium (no filaments)
  - Catenabacterium (filaments)
  - Ramibacterium (false branching)
  - Cillobacterium (motile)

The difficulty is that the characteristic of failure to form spores and that of requiring anaerobic conditions for growth cover an otherwise heterogeneous group of microorganisms, not only these but also anaerobic streptococci, Viellonella, etc., most of which are poorly known, both in their relation to one another and to other microorganisms. In view of this, they will, for present purposes, be discussed here under the single name Bacteroides without generic implication in the taxonomic sense.

Those forms which are present in great numbers in normal feces will grow upon the usual laboratory mediums. Those which are associated with pathological processes in man, however, are nutri-

tively fastidious and require infusion mediums enriched by the addition of blood, yeast, or vegetable extracts, and similar substances, together with glucose and cysteine. In some cases they may be isolated on beef or veal infusion blood agar. Some strains grow in amino acid mediums supplemented with all the known bacterial vitamins; pyruvic acid appears to be of considerable importance in the nutrition of these organisms. The optimum pH is 6.3 to 7.0, and the optimum temperature for growth is 37° C. Completely anaerobic conditions are essential, and growth is favored by the presence of carbon dioxide. *In vitro* they are sensitive to tetracycline and chloramphenicol, but are resistant to streptomycin as well as penicillin.[58]

The better known of these bacteria may be described briefly.

Bacteroides Fusiformis (*Bacillus fusiformis, Fusiformis fusiformis, Fusobacterium plauti-vincenti*). These fusiform bacilli are found in ulcerative stomatitis (trench mouth) and Vincent's angina. Their relation to the spirochetes with which they are usually associated is considered above and elsewhere (Chap. 33). They appear as slender rectilinear or incurving bacilli and frequently assume a filamentous form. They are gram-negative and tend to stain irregularly with the ordinary aniline dyes. Acid but no gas is produced from dextrose, levulose, sucrose, maltose, and sometimes from lactose. They may be isolated on blood agar incubated anaerobically, and the colonies are small and surrounded by a zone of green hemolysis. *Bacteroides fusiformis* is not pathogenic for experimental animals in pure culture, but in mixed culture produces abscesses.

Bacteroides Fragilis (*Bacillus fragilis, Fusiformis fragilis*). This bacterium was found by Veillon and Zuber in twenty-two cases of appendicitis and since has been found in lung, pelvic, and hepatic abscesses, in septicemia with metastatic abscesses, and in infections of the urinary tract. The cells are small, slender rods, sometimes slightly curved. They are gram-negative and nonmotile. *Bact. fragilis* is difficult to isolate but will grow on the usual laboratory mediums. The colonies are small (less than 1 mm.) and transparent. No hemolysis is apparent on blood agar. There is a marked tendency to autolysis with apparent resorption of the colonies, and broth cultures are no longer viable after 7 to 8 days' incubation. A variety of sugars are fermented to acid and gas. The pathogenicity of this bacterium for experimental animals is uncertain.

Bacteroides Funduliformis (*Actinomyces necrophorus, Fusiformis necrophorus, Streptothrix necrophorus, Bacillus necrophorus, Corynebacterium necrophorum,* Schmorl's bacillus). These bacilli have been found in abscesses of the liver, lung, and other parts of the body, in chronic ulcerative colitis, and in the blood stream. Infections with these microorganisms are probably more common than is generally

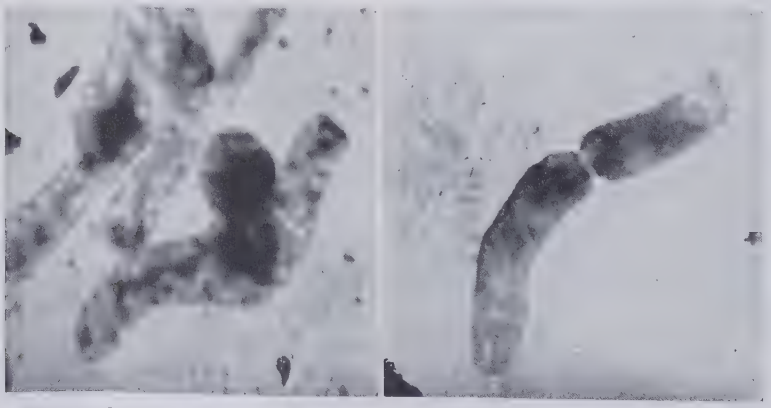


Figure 150. *Bacteroides funduliformis.* Electron micrographs. *Right,* a pair of cells resulting from simple fission, fixed in formalin; × 3300. *Left,* swollen cells containing granular material, especially in the swollen areas, fixed in formalin; × 4900. (Smith, Mudd, and Hillier.[56])

realized. *Bact. funduliformis* has also been found in lower animals as in bovine liver abscesses. The bacilli are highly pleomorphic; slender straight and curved rods may be found intermingled with filamentous and swollen forms, and "ghost cells" which do not stain are frequent. There is a marked tendency to irregular staining, and the bacilli are gram-negative. The colonies on blood agar are variable in size from plate to plate and surrounded by a zone of green hemolysis which may become clear upon prolonged exposure to the air. Glucose, maltose, and levulose are fermented to acid, and there is no evidence of proteolytic activity in gelatin or coagulated egg-white cultures. Some strains give rise to a spreading necrotic lesion upon subcutaneous injection in the rabbit which is usually fatal, while others produce only a localized lesion. Guinea pigs are relatively resistant.

Bacteroides Ramosus (*Fusiformis ramosus, Ramibacterium ramosum, Bacillus ramosus.* Not to be confused with *Bacillus ramosus,* a name sometimes applied to *Bacillus mycoides*). These bacteria are frequently present in the pus of appendicitis and have been encountered in pulmonary gangrene. They appear as small, slender rods which often show branching Y forms and pseudofilaments. They are gram-positive. *Bact. ramosus* is pathogenic for experimental animals, giving rise to abscesses in rabbits and guinea pigs on subcutaneous injection; fatal infections are produced by intravenous inoculation.

Bacteroides Melaninogenicus (*Ristella melaninogenica*). This microorganism has been found in the mouth, tonsils, infected abdominal wounds, focal infections of the kidneys, in stools from patients with chronic amebic dysentery, and in puerperal sepsis. It is described as a very small gram-negative diplobacillus. On blood agar its colonies are black owing to the slow (4 to 5 days) formation of a melanin-like pigment. *Bact. melaninogenicus* grows well in mixed culture but sparsely in pure culture. It is difficult to obtain in pure culture and, when admixed with other bacteria in a colony, colors the entire colony black. Acid is formed from dextrose, levulose, lactose, maltose, sucrose, and mannitol. It is markedly proteolytic and rapidly digests coagulated serum and other native proteins. Its pathogenicity as a primary invader is uncertain.

Bacteroides Pneumosintes (*Dialister pneumosintes*). *Bact. pneumosintes* was described by Olitsky and Gates as the causal agent of influenza. Now known to bear no relation to this disease, its pathogenicity for man is uncertain. It may be cultured from nasopharyngeal washings in Smith-Noguchi medium (human ascitic fluid containing a piece of sterile rabbit kidney and covered with a petrolatum seal) and after a few transfers will grow anaerobically on blood agar, chocolate agar, and Bodet's medium. These bacteria are minute bodies arranged singly, in pairs, or in short chains; they are nonmotile and gram-negative. Because of their small size they pass Berkefeld V and N filters. They produce acid but no gas from dextrose; other sugars are not fermented. If mass cultures are injected intratracheally into rabbits, there is a rise in temperature and sometimes conjunctivitis and mononuclear leucopenia with recovery in 2 or 3 days. If the animal is sacrificed, the lungs are found to be edematous with hemorrhages on the surfaces. *Bact. pneumosintes* is not pathogenic for monkeys.

STREPTOBACILLUS MONILIFORMIS (RAT-BITE FEVER, HAVERHILL FEVER)

A microorganism variously known as *Streptothrix muris-ratti, Haverhillia multiformis, Streptobacillus moniliformis, Actinomyces muris* and *A. muris-ratti* is the cause of an acute febrile disease sometimes given the descriptive name

erythema multiforme. It is also called rat-bite fever because it may be acquired through the bite of infected rats.

For a good many years the etiology of the disease known as rat-bite fever was confused, for Schottmüller, Blake, and others isolated from the blood of patients a fungus to which the name *Streptothrix muris-ratti* was given, while Japanese work indicated that a similar disease, known in Japan as *sodoku,* is caused by a spirochete. In 1925 an epidemic, milk-borne disease with unusual and distinctive clinical features occurred in Haverhill, Massachusetts, and was given the descriptive name *erythema arthriticum epidemicum* or Haverhill fever by Place, Sutton, and Willner.[46] The causative microorganism was isolated by Parker and Hudson;[40] it proved to be a member of the Actinomycetaceae and was given the provisional name *Haverhillia multiformis.* In the same year Levaditi[33] independently studied a similar organism from a case clinically like Haverhill fever and named it *Streptobacillus moniloformis.* Other work has yielded similar results. The Japanese work has also been amply confirmed, and it is now clear that there are two types of rat-bite fever, completely different in etiology.

The microorganism is, like the other actinomycetes, pleomorphic in culture with branching filaments, fragmented, bacillary and coccobacillary forms being found in stained smears. Staining may be irregular, and swollen, and club-shaped cells are found. It has been suggested that this organism is related to the organisms of the pleuropneumonia group. It is not acid-fast and is gram-negative. It requires enriched mediums for growth; Parker and Hudson found whole rabbit blood, allowed to clot and then inactivated, and broth containing serum or ascitic fluid excellent liquid mediums, and glycerin extract of potato, mixed with infusion broth, enriched with egg yolk, and coagulated by inspissation, the best solid medium. Löffler's serum medium is not particularly satisfactory. Growth is markedly better under anaerobic conditions in the presence of added carbon dioxide than in air.

When the disease in man is contracted through a rat bite, the initial wound heals but after a week or 10 days becomes inflamed and painful. Adenitis develops and is followed by toxic symptoms which are the first evidence of the disease acquired from milk. These include headache, chills, vomiting, and general malaise. An eruption of morbilliform character appears, especially on the extremities, and there is multiple arthritis, often severe. The clinical resemblance to rat-bite fever of spirochetal etiology is very close. The causative microorganism may be isolated by blood culture and has been found in the fluid of affected joints. Agglutinins are formed and according to Brown and Nunemaker[4] are of considerable diagnostic utility.

S. moniliformis has been found to be a normal parasite of rats and mice and is present in the nasopharynx. It may assume pathogenicity for the rodent host and produce sporadic or epidemic disease of either an acute septicemic or chronic polyarthritic type. An epizootic of the latter type in wild mice has been reported from Australia. The organism has also been found in bronchopneumonia of rats. Although usually of low virulence on artificial inoculation in rats, an experimental infection has been produced with one strain that gives an osteoarthritis.[32] It is clear that man may acquire the disease by the bite of normal rats, and probably almost all sporadic cases arise in this way. Brown and Nunemaker are of the opinion that rat bite produces the streptobacillus infection much more commonly than the spirochete infection in this country. The number of undiagnosed cases and the proportion of previously reported cases of rat-bite fever that were *S. moniliformis* infections are not known. As indicated above, the Haverhill epidemic was milk-borne; another milk-borne outbreak involving eighty-six persons was reported in 1934. Parker and Hudson found evidence which suggested that the Haverhill milk was contaminated by an infected cow, but this could not be proved; others have suggested contamination of milk by rats as a possibility.

BARTONELLA

Bartonella Bacilliformis. Oroya fever, an infectious anemia, and verruga peruana, a disease characterized by miliary or nodular eruptions, have existed for centuries in certain districts in Peru and recently have been found in Colombia and Ecuador. It was shown by Carrión, through fatal self-inoculation, that the two are stages or manifestations of a single disease which is now commonly known as *Carrión's disease.* The etiological agent is a small pleomorphic bacillus which was observed by Barton in 1905 and named *Bartonella bacilliformis* by Strong, Tyzzer, and Sellards.

Morphology and staining.[45] *Bart. bacilliformis* is a small, motile, aerobic, gram-negative bacillus 0.2 to 0.5 μ in diameter and 1 to 2 μ in length which is found as a slightly curved rod occurring singly, end to end in pairs, and in short chains. A rounded ovoid form, 0.3 to 1 μ in diameter, is also observed singly, in pairs, and in groups. It stains reddish violet with Giemsa's stain, sometimes showing a reddish purple granule at one end of a bluish rod.

Physiology. This microorganism was first cultivated in semisolid leptospira medium and later in tissue culture and in the developing chick embryo, though it could not be carried in serial transfer in the last. Sparse growth occurs on cystine-dextrose-blood agar, and apparently the X factor is required while the V factor is not. Gieman[14] has developed a tryptone-serum medium in both liquid and solid form which supports excellent growth. Little is known of its physiological processes.

Pathogenicity for man.[51] As indicated above, the disease occurs in two forms: the systemic form, in which the red cells are infected; and the histoid cutaneous form. The two may exist independently, coexist, or occur separately, though the usual course is the systemic form followed by the cutaneous form if the former is not fatal, or the cutaneous form alone. The first is a severe, often fatal, febrile anemia. The incubation period is given as about 3 weeks. The anemia is frequently severe, and the loss of red cells may be 200,000 to 300,000 per cu. mm. per day until the total count is half a million or less. The anemia is thought to be due to the direct action of the microorganisms on the erythrocytes, including hemolysis, and to tissue hemorrhages.[50] The fever is of an irregular remittent type, and pain in the bones, joints, and head is common. The case fatality rate is 20 to 40 per cent, death occurring in 2 to 3 weeks after onset.

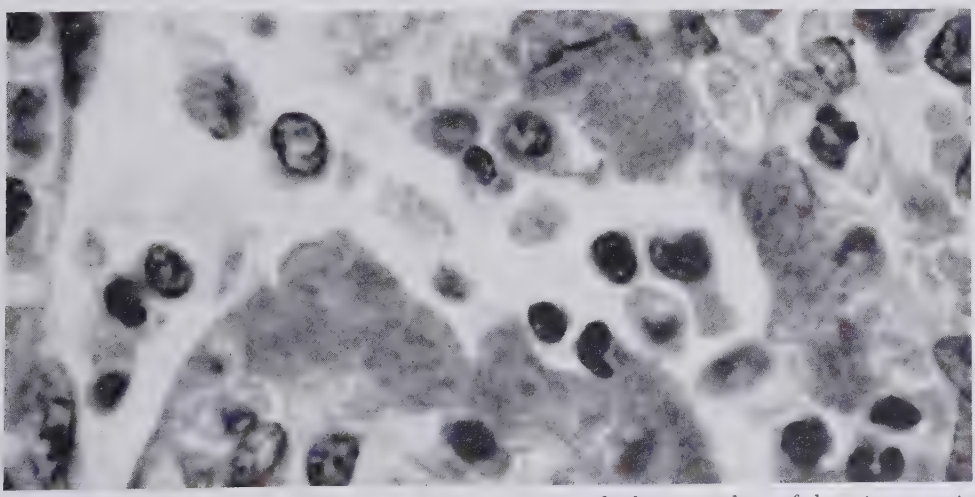


Figure 151. *Bartonella bacilliformis* in human spleen. Note the huge numbers of the microorganisms packed into the lining cells. Giemsa; × 1450. (Humphreys.)

With recovery the eruptive stage of the disease appears and persists for 2 to 3 months. Whether it follows the systemic form or is the primary clinical manifestation of infection, this stage is characterized by a miliary or nodular eruption; the former is by far the more common. The miliary eruption is most common on the face and extremities, appearing as a macule which becomes nodular and eventually disappears leaving no scar. The nodular type of eruption develops more slowly; the nodules may become 2 to 3 cm. in diameter and have a tendency to become strangulated. They are formed by the proliferation of the endothelial cells of the vessels which become obstructed with an inflammatory exudate of plasma cells and fibroblasts and show a marked tendency to hemorrhage.

Bartonella cells are found in large numbers within the erythrocytes in Oroya fever and may be demonstrated in Giemsa-stained blood smears. In both forms of the disease they are found in the tissue macrophages, especially the vascular endothelial cells of the lymphatics, spleen, and liver, often in large clusters within individual cells. The infection is frequently complicated by Salmonella infection to give a high mortality.[6] It is reported that blood cultures are frequently positive in the systemic infection, but it is not clear whether this is a reliable method of diagnosis. The disease has been treated successfully with chloramphenicol and tetracycline.[44, 60, 67]

Epidemiology. Bartonellosis is strictly limited geographically, being, so far as is known, exclusively American and tropical, occurring in the Andes between latitudes 2° N. and 13° S. It is transmitted by *Phlebotomus verrucarum* and *P. noguchii* in Peru; whether other species of Phlebotomus or other arthropods, such as Dermacentor, are natural vectors is not known. Peruvian ground squirrels (*Citellus tridecemlineatus*) have been infected experimentally[27] and, together with domestic animals, including chickens, and guinea pigs and field mice, may possibly be naturally infected. Whether there is such an animal reservoir of infection, or the disease is maintained in human infections only, is not known.

Immunity. It is generally said that recovery from an attack of either form of the disease confers a solid immunity to both. With the cultivation of *Bart. bacilliformis*, it has been possible to study the occurrence of circulating antibody, agglutinins, and investigate the possibilities of prophylactic inoculation. Agglutinins may be demonstrated during the early stages of the disease but, in spite of the lasting immunity, almost always disappear with the subsidence of clinical symptoms. Their diagnostic significance is not as yet known.

Pathogenicity for animals. In general experimental animals appear to be highly resistant to infection with *Bart. bacilliformis*. Both the eruptive and systemic forms of the disease have been produced in rhesus monkeys, the latter form in splenectomized animals.

Bartonellosis of Animals. Naturally occurring bartonellosis has been found in a number of animals, including the dog, cattle, and a variety of rodents. The infection takes a systemic rather than eruptive form and is usually latent but activated when host resistance is reduced as by splenectomy. The bartonella-like organisms of lower animals have been separated into three genera: Haemobartonella, Grahamella containing but a single species (*Grahamella talpae*) which differs from Haemobartonella in that the infection is not eradicated by treatment with arsenicals; and Eperythrozoon, which is more highly pleomorphic than the first two groups and is found in the plasma as well as in the erythrocytes. None of these forms has been cultivated, and all appear to be nonpathogenic for man.

Of these, the most commonly encountered is *Haemobartonella muris* which infects rats and is transmitted by the rat louse, Haematopinus, and the rat flea, *Xenopsylla cheopsis*. The infection is very common, and the great majority of laboratory rats are infected. The infec-

tion is latent and may be precipitated in an acute form by splenectomy with the appearance of parasitized red cells in the circulating blood. Special strains of rats are maintained bartonella-free for experimental purposes. *H. canis* is the cause of an infectious anemia of dogs and is transmitted by the dog flea, Ctenocephalus. *H. tyzzeri* occurs in guinea pigs, *H. microtii* in the vole, *H. bovis* in cattle, and other species in mice, shrews, and squirrels.

Eperythrozoonosis similarly occurs as a latent infection which is activated by splenectomy. *Eperythrozoon coccoides* is a parasite of white mice, and other species occur in wild mice. The disease also occurs in sheep and cattle, and the causative organisms are *E. ovis* and *E. wenyonii* respectively.

The Systemic Position of Bartonella. The relationship of these microorganisms to the bacteria on the one hand and to the rickettsiae and viruses on the other is of some interest. Among the bacteria the tendency to parasitize the cells of the host and appear as intracellular clumps of microorganisms is most marked in *Past. tularensis* which commonly is found in an intracellular position. Bartonella shows a much more marked preference for intracellular parasitism, and both in this respect and in morphology seems to be closely related to the rickettsiae. It is generally agreed, however, that the relationship is not sufficiently close to justify their classification as rickettsiae, and they may be considered as lying between them and the bacteria.

THE PLEUROPNEUMONIA-LIKE ORGANISMS[10, 31, 53, 59] (MYCOPLASMA)

The first of an apparently more or less homogeneous group of highly pleomorphic, filtrable microorganisms to be described is the causative agent of *bovine pleuropneumonia.* Subsequently a number of similar forms, both parasitic and saprophytic, have been found. They have been called the pleuropneumonia group or pleuropneumonia-like organisms, though none of the rest produces pleuropneumonia in cattle. They are set rather sharply apart from other microorganisms, and are considered to fall into a single genus, Mycoplasma, and the bovine pleuropneumonia organism is taken to be the type species, *Mycoplasma mycoides.*[11]

Morphology and Staining. There are probably few if any other microorganisms which are as highly pleomorphic as these forms. Impression preparations of colonies show granules of various sizes, filaments which may be branched and contain streaming protoplasm, balloon and disc-like structures, ring, club, and star forms, and ameboid structures. Filaments are more numerous in recently isolated cultures and tend not to be formed in older stock cultures. The microscopic morphology of these forms is illustrated in the accompanying figures. The marked pleomorphism is due in part to the fragility of the organisms, many of which are torn apart in making smears, and, as in the case of *Bact. funduliformis,* in part a consequence of modes of reproduction other than binary fission, *e.g.,* that of the development of round bodies which become nodular with outgrowths that segment into daughter cells. This process has been studied in detail by means of electron micrographs. Viable structures vary greatly in size and include ultramicroscopic elements which are filterable. Experiments on filtration through gradocol membranes have been described in which the concentration of organisms in an emulsion was reduced from 10^8 to 10^5 by passage through a membrane of APD 0.8 μ. This titer decreased progressively with membranes of decreasing pore size, but complete retention did not occur until a membrane of 0.33 μ was used. In contrast to this, the end point is usually sharp in the filtration of viruses. On the basis of these

Figure 152. Two-day culture of type *A* pleuropneumonia-like organism from mice in serum-dextrose broth. Note the rings and elementary bodies. Giemsa; × 1000. (Sabin: Bact. Rev.)

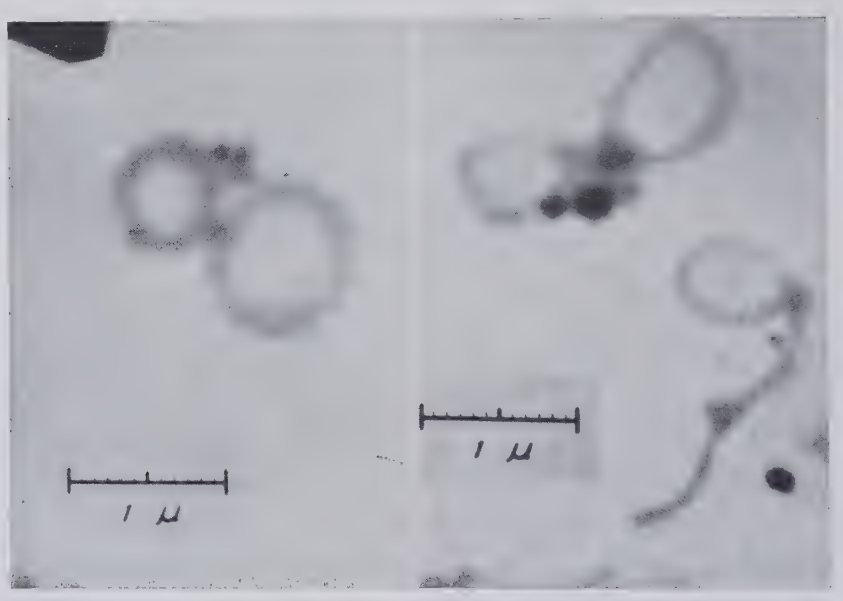


Figure 153. Electron micrographs of the ring forms of the pleuropneumonia-like organisms. (Lilly Research Laboratories.)

observations the diameter of the smallest viable elements was estimated to be 165 to 247 mμ. Similar estimates vary somewhat for other strains; the agent of pleuropneumonia, for example, is 125 to 175 mμ.

These forms cannot be found in tissues by any method of staining. They are not demonstrable in smears stained by the usual aniline dyes, but are stained by certain polychrome stains, Giemsa, and Castaneda's rickettsia stain. Very thick films prepared from the sediment of centrifuged cultures are gram-negative.

On primary isolation or change to a slightly different culture medium, broth cultures sometimes, but not always, show no detectable evidence of growth and may be carried along by "blind passage" for several transfers before it appears. Some strains show a uniform opalescence, others a granular type of growth. The characteristic of some strains to grow as small colonies, appearing as flakes attached to the side of the tube, is of some differential value. In any case, visible evidence of growth is very slight, and almost all workers with these organisms

have found it necessary to carry along an uninoculated tube of medium for comparative purposes.

The colonies upon an agar surface are usually not detectable until after 2 or 3 days' incubation. They are most readily observed in stained agar preparations. A square of agar is cut from a suspicious area on the plate and placed on a slide. It is covered with a cover slip on which an alcoholic solution of methylene blue and azure has been dried, and the space between the cover slip and the slide filled with melted paraffin. The average colony size of different strains ranges from 0.01 to 0.6 mm., rarely exceeding the larger figure. The colonies usually have a dark center with a lighter margin and may appear coarsely or finely granular. Upon higher magnification a foam-like structure may be observed which is composed of the balloon-like forms. Oily droplets may be found in the colonies of some strains; these have been found, in some cases at least, to be largely cholesterol already present in the medium. Possibly the pseudo colonies which have been observed on uninoculated serum agar are of similar nature; it is said that these are readily differentiated from true colonies by experienced observers.

Physiology.[1] These microorganisms require infusion or digest mediums enriched by the addition of serum or ascitic fluid in relatively large amounts. Their growth requirements, especially on primary isolation, have been studied by Morton and his co-workers.[37, 38] They have shown that these microorganisms are regularly cultivable on a beef heart infusion medium at pH 7.8, containing Bacto peptone or Parke, Davis bacteriological peptone, and enriched with a heat-stable factor present in ascitic fluid and serum. In some cases these organisms tolerate a relatively wide pH range, but others die out at pH 7.0 and below and require a pH of 7.8 to 8.0 for growth. The saprophytic varieties will grow at 22° C. with an optimum at 30° C., but the parasitic ones require 37° C. Growth occurs both aerobically and anaerobically but is less abundant with most strains under anaerobic conditions. Fairly heavy inoculums are required, 0.1 to 0.2 ml. of minced tissue in primary isolation, the transfer of similar amounts of broth cultures, and in transfer from agar cultures a small section of the medium is cut out and dropped into liquid mediums or streaked on another plate. These organisms may also be cultured on the chorioallantoic membrane of the developing hen's egg. Some sugars, notably glucose, are fermented, but the pH seldom drops below 7.0 owing to the death of the organisms at this point; the addition of other sugars may inhibit growth. In general, fermentations and other biochemical characteristics have no differential value. The heat resistance of some strains is of the same order as that of most bacteria, while others appear to be more frail and are killed by exposure to

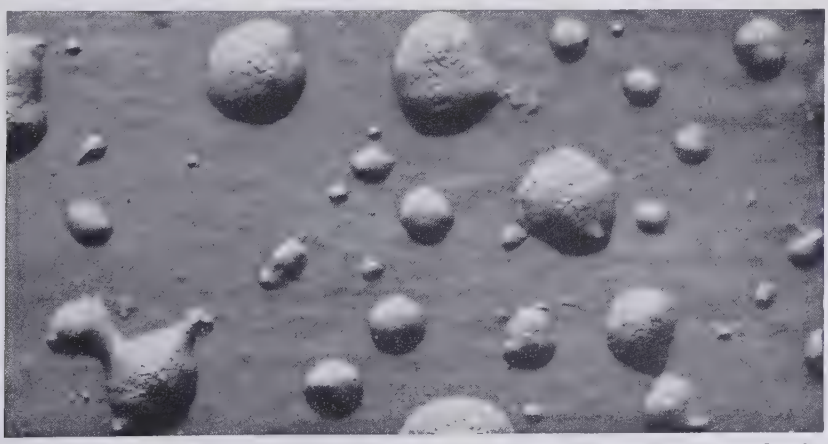


Figure 154. Colonies of a type B pleuropneumonia-like organism from mice. Three days' incubation: × 100. (Sabin: Bact. Rev.)

Figure 155. The reproductive process of pleuropneumonia-like organisms. Both small round cells and rod-shaped cells, some of which have rounded enlargements, are shown. The round bodies develop from the rod-shaped cells in this way (*lower left*). The round bodies enlarge, and the surface becomes nodular (*lower center*), and outgrowths occur and segment into multiple daughter cells (*lower right*). The round bodies are not spores and do not have the characteristic spore cell wall. Preparations fixed in osmic acid; × 15,000. (Rearranged from Smith, Hillier, and Mudd.)

45° C. for 15 minutes. Cultures are best preserved at incubator temperature in mediums containing no added sugar; when sealed with petrolatum the organism may remain viable for a month or more.

Varieties, Strains, or Species. Strains of these organisms have been isolated from a variety of sources. Those which are parasitic are definitely set off from the saprophytic types, and differentiation or identification within these groups has been made in part on source and pathogenicity and in part on an immunological (agglutination) basis.

With the study of bacterial protoplasts and their similarities to both the L forms of bacteria and the pleuropneumonia-like organisms (PPLO), and the association of the last two with one another (see below), the existence of the PPLO as a group of independent entities becomes uncertain. It has been urged by a number of workers, especially Dienes, that the PPLO represent stages in the life history of microorganisms better known in other forms. While there is no convincing evidence that an orderly, cyclical transformation of morphological types occurs among bacteria in this sense (Chap. 7), at the same time the possibility that the PPLO are naturally occurring protoplasts, differing from the parent microorganism only in lacking a cell wall structure, is highly suggestive. While, then, the PPLO are considered to be independent entities, and given the generic name Mycoplasma in a separate order Mycoplasmatales, any attempt to do other than describe these forms, their origin, and pathological conditions with which they have been associated seems premature.

Bovine pleuropneumonia. As indicated above, the causative organism of pleuropneumonia of cattle was the first of these forms to be isolated and studied. The disease is found all over the world except in India, Western Europe, and North America; it has been imported into this country a number of times but was finally eradicated by slaughter of infected animals and has not occurred since 1892.

The natural disease in cattle is characterized by extensive consolidation and

subpleural effusion in either or both lungs, and the microorganism is present in large numbers in the serous exudate. It spreads slowly in herds and may take an acute form with death within a week or a chronic form with walling off of the foci of infection. There is occasional joint involvement in young animals. The natural disease has not been reproduced in cattle by either inoculation of infectious serous exudate or of cultures, but an extensive edema develops and spreads from the site of inoculation, and there is a febrile reaction and sometimes death.

The organism is apparently completely nonpathogenic for the usual experimental animals and for man; it has been reported that cultures in sheep- or horse-serum mediums are infectious for sheep and goats, while those in bovine-serum mediums are not.

The name *Asterococcus mycoides* was given the organism by Borrel and Dujardin-Beaumetz but has not been generally used. In any case, strains obtained in different localities and at different times appear to be very similar if not identical.

Contagious agalactia of sheep and goats. Despite its name this disease is a generalized infection which affects both males and females. It occurs only in parts of southern Europe and North Africa; agalactia or mastitis of these animals in this country is of other etiology.

The lesions occur in the joints and eyes and in the mammary glands of females. The microorganism is present in the blood early in the disease and later may be isolated from the affected regions and from the mammary secretions. The second member of the group to be studied, it was isolated by Bridré and Donatien in 1923. It is morphologically and biochemically very similar to the pleuropneumonia organism and other members of the group but is immunologically distinct and of characteristic pathogenicity. The disease is readily reproduced by inoculation of sheep and goats with cultures.

Canine variety. Organisms of the pleuropneumonia group have also been found in dogs. Shoetensak reported in 1934 the cultivation of an organism of this type from the purulent nasal discharge of dogs ill with distemper which he called *Asterococcus canis*. In later studies other organisms were found and there appeared to be two types, immunologically distinct from one another, which he called type I and type II respectively. Their postulated etiological relationship to distemper, generally regarded as a virus disease, is not established.

Varieties from rats. A series of pleuropneumonia-like organisms has been isolated by Klieneberger and her associates from the respiratory tract and elsewhere in normal and diseased rats. Others have isolated similar organisms from rats exhibiting polyarthritis and swollen extermities. These comprise, with a single exception, the "L" series of strains in which the strains are designated by subscript numerals. There now appear to be three distinct types, differentiated on a biological and immunological basis, *viz.*, L_1, L_3, and L_4. The strain L_2 was isolated from a guinea pig and insufficiently studied before loss of the culture at the outbreak of war. The strain described as L_5 is immunologically identical with mouse type A discussed below; L_6 has been insufficiently studied; and L_7 has been found to be identical with L_4.

Considerable interest has attached to the apparent association of L_1 with *S. moniliformis*. Klineberger-Nobel has been able to isolate L_1 from many strains and stock cultures of *S. moniliformis* and it will be recalled that this organism is a natural parasite of the nasopharynx of rats and mice. It is maintained by Klieneberger-Nobel that L_1 and streptobacillus coexist as symbionts since she has been able to separate the two on the basis of differential resistance to heat and aging, has observed L_1 in the rat in the absence of *S. moniliformis*, and has carried L_1 strains through many transplants without its reappearance.

These L forms are associated, causally in some instances at least, with relatively mild, chronic affections of rats in which a not uncommon manifestation is joint involvement and polyarthritis. They closely

resemble the other organisms of the group morphologically and culturally, forming a subgroup on the basis of pathogenicity, and differentiated from one another immunologically.

Varieties from mice. Sabin divides the pleuropneumonia-like organisms found in mice into five types, designated as A, B, C, D, and E. Type A is found in normal mice, once in the brain and frequently in the eyes, nasal mucosa, and lungs of carriers. On intracerebral inoculation of mice an ataxia is produced which is characterized by a turning or rolling of the body. The brain lesions and symptoms arising from them are attributable to the action of a soluble toxin produced by the microorganisms. An immunologically identical organism was isolated by Findlay *et al.* from mice affected with "rolling disease" and designated L_5. Type B has likewise been found in normal mice and is not only immunologically distinct but produces a progressive arthritis almost exclusively when parenterally inoculated into mice. Types C, D, and E are similar in their pathogenicity but are immunologically distinct; they have not been studied as extensively as some of the others.

Pleuropneumonia-like organisms of man. Though there is considerable evidence suggesting that certain streptococci are associated with some types of arthritis in man, the etiology of human arthritis is far from clear. The pleuropneumonia-like organisms have assumed some interest in this connection, largely because of the frequency of occurrence of progressive polyarthritis in both naturally and artificially infected rats and mice. The association of L_1 strains with *S. moniliformis* is of possible interest in view of the arthritic manifestations of epidemic disease of the Haverhill fever type and of sporadic rat-bite fever of fungous etiology.

Microorganisms of this group have been found in the genital tract by a number of workers. They appear to occur with some frequency in the normal vagina and cervix, in 58 of 222 routine specimens in a series, but they have also been found associated with inflammatory conditions of the cervix. In the male, however, they have been found largely in association with pathology of the genitourinary tract. All of the fifty-eight patients from whom cultures were recovered had urethritis, prostatitis, or cystitis. In relation to the possible association of these microorganisms with arthritis in man, it is of interest that eighteen of this series had an acute type of arthritis, nine had simultaneous urethritis, conjunctivitis, and arthritis, the syndrome characteristic of Reiter's disease, and in two instances pleuropneumonia-like organisms were recovered from synovial fluid. Their pathogenicity is not unequivocally established, but their association with conditions such as nongonococcal urethritis (Chap. 19) may be regarded as suggestive.

Saprophytic varieties. Pleuropneumonia-like organisms presumably living a saprophytic existence in nature have been found by Laidlaw and Elford in raw London sewage. They closely resembled the parasitic forms both morphologically and culturally and fell into three immunological groups which were designated types A, B, and C. All were nonpathogenic for experimental animals. Similar forms have been found in Germany in compost and other types of decomposing organic matter.

DONOVANIA GRANULOMATIS[49]

The disease granuloma inguinale (granuloma venereum) is not to be confused with lymphogranuloma inguinale of virus etiology. It is characterized by a slowly progressive ulceration in the genital region and rarely elsewhere. The initial lesion is a swelling, often in the groin as a bubo, which ruptures. Daugh-

ter lesions appear which are at first discrete and then spread slowly and coalesce, and the process may eventually involve the skin of the groin, genitals, buttocks, and lower abdomen, and the patient develops a strong fetid odor. Little effective immunity appears to be developed, at least not sufficient to appreciably arrest the progress of the infection.

Bacillary bodies, stained by Wright's stain, were observed by Donovan in 1905 in smears from lesions or in biopsy material, and have long been known as Donovan bodies. They may also be stained with 1 per cent pinacyanole in methanol, the capsules assuming a purplish pink and the cell body a dark blue color. The Donovan body has been cultivated in the yolk sac, but not on the chorioallantois, of the developing chick embryo and in enriched mediums such as beef heart infusion[20] and is thus shown to be a cultivable microorganism to which the name *Donovania granulomatis* has been given.[16, 17]

It is a short, plump bacillus 1.5 μ to 4.5 μ in length and 0.8 μ to 1.4 μ in breadth, gram-negative and showing prominent polar granules. Prior to cell division the elongated bacillary forms tend to become curved and may remain attached after division to give rise to chains of bacilli, the coiled filaments often seen in the usual stained preparations. The relatively heavy encapsulation observed in preparations from lesion material and the mucoid character of yolk sac cultures and initial cultures on artificial mediums diminish with continued culture. Aside from its highly fastidious growth requirements, *D. granulomatis* closely resembles Friedländer's bacillus and has been found to be closely related immunologically to this bacterium and to other coliform bacilli. There are antigenic differences between strains, complicating the problem of serodiagnosis, as by complement fixation.[15, 18]

Prior to the isolation in pure culture of *D. granulomatis*, its causal relation to granuloma inguinale was only suggested by association. Material from yolk sac cultures gives a skin reaction in persons having the disease, and a mucoid substance from infected yolk sac, possibly a polysaccharide, gives precipitin and complement fixation reactions with serums from patients. The specificity of these reactions is, however, open to question in that apparently specific complement fixation was given by persons with chronic, nonspecific ulceration perhaps attributable to the serological relation of *D. granulomatis* to coliform bacilli. Greenblatt and his co-workers were able to produce the disease in two human volunteers, one inoculated by a subcutaneous transplant of biopsy material and the other by the subcutaneous inoculation of yolk sac culture, but later were unable to repeat these results.[9] Since it has not yet

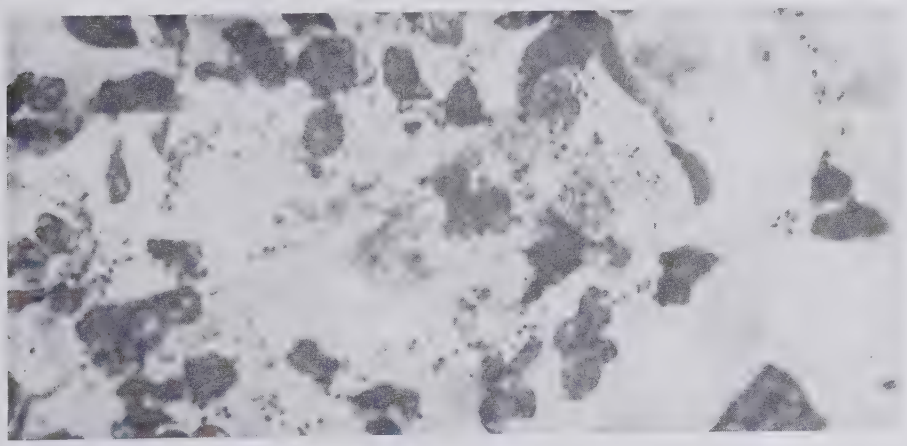


Figure 156. *Donovania granulomatis* in a vaginal smear. The microorganisms are the small ovoid bodies both within polymorphonuclear leucocytes and lying free. Wright's stain; × 1200.

been possible to infect experimental animals other than the chick embryo, the etiological relation of *D. granulomatis* to granuloma inguinale cannot be regarded as fully established. Successful chemotherapy of the disease with chloramphenicol and tetracyclines has, however, been reported.

The general tendency to regard this disease as venereal is based in large part on the location of the lesions. There is little or no direct evidence that it is transmitted primarily by sexual contact and, in fact, its occurrence in both marital partners is uncommon. The probable incubation period of 1 to 4 weeks is not excessively long and should not greatly obscure histories of contact. It has been suggested that there is great individual variation in susceptibility and that natural resistance is usually of a high order. The disease is associated with uncleanliness and is found in the Far East, Africa, and the Americas. In this country it occurs for the most part in the Negro of low economic status in the southeastern states, but it occurs elsewhere also. It is estimated that there are 5000 to 10,000 cases in the United States, and it has been found to constitute 2 to 3 per cent of venereal disease in Negro recruits. In general, however, the epidemiology of granuloma inguinale is as yet very poorly understood.

REFERENCES

1. Adler, H. E., and Shifrine, M.: Nutrition, metabolism, and pathogenicity of Mycoplasmas. Ann. Rev. Microbiol., 1960, **14:**141–160.
2. Andrieu, G., Monnier, J., and Bourse, R.: La sensibilité de bacille pyocyanique aux agents anti-bactèriens d'utilisation clinique. Ann. Inst. Pasteur, 1957, **92:**176–189; 322–334.
3. Beigelman, P. M., and Rantz, L. A.: Clinical significance of Bacteroides. Arch. Intern. Med., 1949, **84:**605–631.
4. Brown, T. McP., and Nunemaker, J. C.: Ratbite fever: A review of the American cases with re-evaluation of etiology; report of cases. Bull. Johns Hopkins Hosp., 1942, **70:**201–328.
5. Claypool, B. W., Jr., Wellman, W. E., Needham, G. M., and Achor, R. W. P.: *Listeria monocytogenes* meningitis: Brief review of the literature and report of a case. Proc. Staff Meetings Mayo Clinic, 1960, **35:**609–614.
6. Cuadra, O. M.: Salmonellosis complication in human bartonellosis. Texas Repts. Biol. Med., 1956, **14:**97–113.
7. Curtin, J. A., Petersdorf, R. G., and Bennett, I. L., Jr.: Pseudomonas bacteremia: Review of ninety-one cases. Ann. Intern. Med., 1961, **54:** 1077–1107.
8. Dack, G. M.: Non-sporeforming anaerobic bacteria of medical importance. Bact. Rev., 1940, **4:**227–258.
9. Dienst, R. B., Chen, C. H., and Greenblatt, R. B.: Experimental studies on the pathogenicity of *Donovania granulomatis*. Amer. J. Syph., 1949, **33:**152–157.
10. Edward, D. G. ff.: The pleuropneumonia group of organisms: A review, together with some new observations. J. Gen. Microbiol., 1954, **10:**27–64.
11. Edward, D. G. ff., and Freundt, E. A.: The classification and nomenclature of organisms of the pleuropneumonia group. J. Gen. Microbiol., 1956, **14:**197–207.
12. Elrod, R. P., and Braun, A. C.: *Pseudomonas aeruginosa*: Its role as a plant pathogen. J. Bact., 1942, **44:**633–643.
13. Fisher, A. M., and McKusick, V. A.: Bacteroides infections. Clinical, bacteriological and therapeutic features of fourteen cases. Amer. J. Med. Sci., 1953, **225:**253–273.
14. Gieman, Q. M.: New media for the growth of *Bartonella bacilliformis*. Proc. Soc. Exptl. Biol. Med., 1941, **47:**329–332.
15. Goldberg, J.: Studies on granuloma inguinale. III. The antigenic heterogeneity of *Donovania granulomatis*. Amer. J. Syph., 1954, **38:** 330–335.
16. Goldberg, J.: Studies on granuloma inguinale. IV. Growth requirements of *Donovania granulomatis* and its relationship to the natural habitat of the organism. Brit. J. Venereal Dis., 1959, **35:**266–268.
17. Goldberg, J., Weaver, R. H., and Packer, H.: Studies on granuloma inguinale. I. Bacteriologic behavior of *Donovania granulomatis*. Amer. J. Syph., 1953, **37:**60–70.
18. Goldberg, J., Weaver, R. H., Packer, H., and Simpson, W. G.: Studies on granuloma inguinale. II. The complement fixation test in the diagnosis of granuloma inguinale. Amer. J. Syph., 1953, **37:**71–76.
19. Gray, M. L.: Isolation of *Listeria monocytogenes* from oat silage. Science, 1960, **132:** 1767–1768.
20. Hall, W. K., Dienst, R. B., and Chen, C. H.: A growth factor required by *Donovania granulomatis*. Proc. Soc. Exptl. Biol. Med., 1953, **84:**370.
21. Harrison, R. W.: Studies on lactobacilli. III. Relationship of immunological specificity and fermentative capacity. J. Infect. Dis., 1942, **70:**77–87.
22. Harrison, R. W.: Oral microbiology. Ann. Rev. Microbiol., 1949, **3:**317–330.
23. Harrison, R. W., and Opal, Z. Z.: Comparative studies on lactobacilli isolated from the mouth and intestine. J. Dental Res., 1944, **23:**1–22.
24. Harrison, R. W., Zidek, Z. C., and Hemmens, E. S.: Studies on lactobacilli. II. Type-specific immunological reactions of oral strains. J. Infect. Dis., 1939, **65:**255–262.
25. Haynes, W. C.: *Pseudamonas aeruginosa*—its characterization and identification. J. Gen. Microbiol., 1951, **5:**939–950.
26. Henrich, S., and Pulverer, G.: Über den Nachweis des *Bacteriodes melaninogenicus* in

Krankheitsprozessen bei Mensch und Tier. Ztschr. f. Hyg. u. Infektionskr., 1960, **146:**331–340.
27. Herrer, A.: Carrión's disease. III. Experimental infection of squirrels. Amer. J. Trop. Med. Hyg., 1953, **2:**650–654.
28. Hoffman, H.: Oral microbiology. Ann. Rev. Microbiol., 1957, **11:**183–198.
29. Hunter, C. A., and Ensign, P. R.: An epidemic of diarrhoea in a new-born nursery caused by *Pseudomonas aeruginosa*. Amer. J. Pub. Health, 1947, **37:**1166–1169.
30. Klauder, J. V.: *Erysipelothrix rhusiopathiae* infection in animals and in human beings. Ann. N. Y. Acad. Sci., 1947, **48:**535–552.
31. Kleineberger-Nobel, E.: Pleuropneumonia-like Organisms (PPLO) Mycoplasmataceae. Academic Press, New York. 1961.
32. Lerner, E. M., and Silverstein, E.: Experimental infection of rats with *Streptobacillus moniliformis*. Science, 1957, **126:**208–209.
33. Levaditi, C., Nicolau, S., and Poincioux, P.: Recherches sur l'étiologie de l'érythème polymorphe aign: son agent étiologique: *Streptobacillus moniliformis*. Presse Med., 1926, **34:** 340–343.
34. Mayr-Harting, A.: The serology of *Pseudomonas pyocyaneus*. J. Gen. Microbiol., 1948, **2:**31–39.
35. Moragues, V., and Anderson, W. A. D.: Endocarditis due to *Pseudomonas aeruginosa*. Ann. Intern. Med., 1943, **19:**146–154.
36. Morton, H. E., Smith, P. F., and Keller, R.: Prevalence of pleuropneumonialike organisms and the evaluation of media and methods for their isolation from clinical material. Amer. J. Pub. Health, 1952, **42:**913–925.
37. Morton, H. E., Smith, P. F., and Leberman, P. R.: Investigation of the cultivation of pleuropneumonialike organisms of man. Amer. J. Syph., 1951, **35:**361–369.
38. Orland, F. J.: A correlation of antigenic characteristics among certain bacteria of the lactobacillus group. J. Infect. Dis., 1950, **86:**63–80.
39. Orland, F. J., et al.: Use of germfree animal technic in study of experimental dental caries; basic observations on rats reared free of all microorganisms. J. Dental Res., 1954, **33:** 147–174.
40. Parker, F., Jr. and Hudson, N. P.: The etiology of Haverhill fever (erythema arthriticum epidemicum). Amer. J. Path., 1926, **2:**357–379.
41. Pastor, B. H., Gefter, W. I. and Baer, S.: *Pseudomonas aeruginosa* meningitis following spinal anesthesia: Report of two successfully treated cases. Ann. Intern. Med., 1951, **35:** 1137–1143.
42. Patocka, F., Mara, M. and Schindler, J.: Studies on the pathogenicity of *Listeria monocytogenes*. II. Influence of substances isolated from cells of *Listeria monocytogenes* on experimental listeriosis in white mice. Zent. f. Bakt., I Abt., Orig., 1959, **174:**586–593.
43. Patocka, F., Schindler, J. and Mara, M.: Studies on the pathogenicity of *Listeria monocytogenes*. I. Protein substance isolated from cells of *Listeria monocytogenes* enhancing listeric infection. Zent. f. Bakt., I Abt., Orig., 1959, **174:**573–585.
44. Payne, E. H., and Urteaga, O.: Carrión's disease treated with chloromycetin. Antibiot. Chemotherapy, 1951, **1:**92–99.
45. Peters, D., and Wigand, R.: Bartonellaceae. Bact. Rev., 1955, **19:**150–155.
46. Place, E. H., Sutton, L. E., and Willner, O.: Erythema arthriticum epidemicum. Boston Med. Surg. J., 1926, **194:**285–287.
47. Prévot, A. R.: Manuel de Classification et de Détermination des Bactèries Anaérobies. Monographies d l'Institut Pasteur. Masson et Cie., Paris, 1940.
48. Price, J. E. L., and Bennett, W. E. J.: The erysipeloid of Rosenbach. Brit. Med. J., 1951, **ii:**1060–1062.
49. Rajam, R. V. and Rangiah, P. N.: Donovanosis (granuloma inguinale, granuloma venereum). W. H. O. Monograph Series No. 24. World Health Organization, Palais des Nations, Geneva, 1954.
50. Reynafarje, C., and Ramos, J.: The hemolytic anemia of human bartonellosis. Blood, 1961, **17:**562–578.
51. Ricketts, W. E.: Clinical manifestations of Carrión's disease. Arch. Intern. Med., 1949, **84:** 751–781.
52. Rosebury, T.: Role of infection in periodontal disease. Oral Surg. Oral Med. Oral Path., 1952, **5:**363–370.
53. Sabin, A. B.: The filtrable microorganisms of the pleuropneumonia group. Bact. Rev., 1941, **5:** 1–68.
54. Salvin, S. B., and Lewis, M. L.: External otitis with additional studies on the genus Pseudamonas. J. Bact., 1946, **51:**495–505.
55. Seeliger, H. P. R.: Listeriosis. Hafner, New York. 1961.
56. Smith, W. E., Mudd, S., and Hillier, J.: L-type variation and bacterial reproduction by large bodies as seen in electron micrograph studies of *Bacteroides funduliformis*. J. Bact., 1948, **56:**603–618.
57. Sneath, P. H. A., Abbott, J. D., and Cunliffe, A. C.: The bacteriology of erysipeloid. Brit. Med. J., 1951, **ii:**1063–1066.
58. Stevens, W. C., and Harrison, A. P., Jr.: In vitro antibiotic sensitivities of Bacteroides and similar forms. Antibiot. Chemotherapy, 1958, **8:**192–194.
59. Symposium: Biology of the pleuropneumonialike organisms. Ann. N. Y. Acad. Sci., 1960, **79:** 305–758.
60. Urteaga, B. O., and Payne, E. H.: Treatment of the acute febrile phase of Carrión's disease with chloramphenicol. Amer. J. Trop. Med. Hyg., 1955, **4:**507–511.
61. Verder, E., and Evans, J.: A proposed antigenic schema for the identification of strains of *Pseudomonas aeruginosa*. J. Infect. Dis., 1961, **109:**183–193.
62. Welshimer, H. J., and Wingelwish, N. G.: Listeriosis—summary of seven cases of Listeria meningitis. J. Amer. Med. Assn., 1959, **171:** 1319–1323.
63. White, T. G., and Shuman, R. D.: Fermentation reactions of *Erysipelothrix rhusiopathiae*. J. Bact., 1961, **82:**595–599.
64. Williams, N. B.: Antigenic components of lactobacilli of human origin. J. Infect. Dis., 1948, **82:**31–41.
65. Williams, N. B., Norris, R. F., and György, P.: Antigenic and cultural relationships of *Lactobacillus bifidus* and *Lactobacillus parabifidus*. J. Infect. Dis., 1953, **92:**121–131.
66. Woodbine, M.: *Erysipelothrix rhusiopathiae* bacteriology and chemotherapy. Bact. Rev., 1950, **14:**161–178.
67. Zegarra Araujo, N.: Bartonellosis and tetracycline. Antibiotic Med., 1955, **1:**201–209.

Chapter 28

BACILLUS—THE SPORE-FORMING AEROBES

THE SPORE-FORMING rod-shaped bacteria are divided into two groups on the basis of their relation to atmospheric oxygen. The genus Bacillus includes the aerobic forms, and the anaerobic types are designated Clostridium. A very large number of species of Bacillus have been described, the majority of them from soil and dust. Two species, *Bacillus alvei* and *B. para-alvei,* cause foul brood, a disease of bees. *B. subtilis* infects human beings only rarely, and, with this exception, *B. anthracis* is the only member of this large group that is pathogenic for man.

BACILLUS ANTHRACIS

Primarily a disease of lower animals transmissible to man, anthrax is of particular historical interest, for it was in his study of this disease that Koch provided the first demonstration of the causal relation between a specific bacterium and an infectious disease. The bacillus had been observed in the blood and organs of animals dying of anthrax by Davaine and Rayer in 1850 and by Pollender in 1855. In 1857 Brauell transmitted the disease by the inoculation of blood from infected animals. Conclusive demonstration of the causal relation between the bacilli and the disease, however, was the work of Koch, who in 1877 cultured the bacillus on the aqueous humor of the ox's eye, described its life history, and reproduced the disease with a pure culture of the microorganism. The importance of this discovery to the development of bacteriology has been discussed elsewhere.

Morphology and Staining. The anthrax bacillus is one of the largest of the pathogenic bacteria and ranges from 4.5 to 10 μ in length and from 1 to 1.25 μ in breadth. The ends of the rods are often concave and somewhat swollen so that the appearance of a chain of anthrax bacilli has been compared to a jointed bamboo fishing rod. The cells occur singly and as end-to-end pairs or short chains in the body, but in culture long chains are formed. Unlike most of the sporulating aerobic bacilli they are nonmotile.

Capsules may be found on the bacilli in smears from an infected animal but are not found in culture except on mediums rich in animal protein, such as serum agar. The capsular material is not polysaccharide as it is in most bacteria, but is a high molecular weight polypeptide composed exclusively of *d*(-) glutamic acid (the "abnormal" stereoisomer).[25, 50] This is a point of particular interest, for it is the first demonstrated natural occurrence of *d*(-) glutamic acid and of a polypeptide composed of a single amino acid. There is, in addition, a polysaccharide haptene present in the cell substance of the bacilli, which may be isolated from these bacilli;[35] it appears to

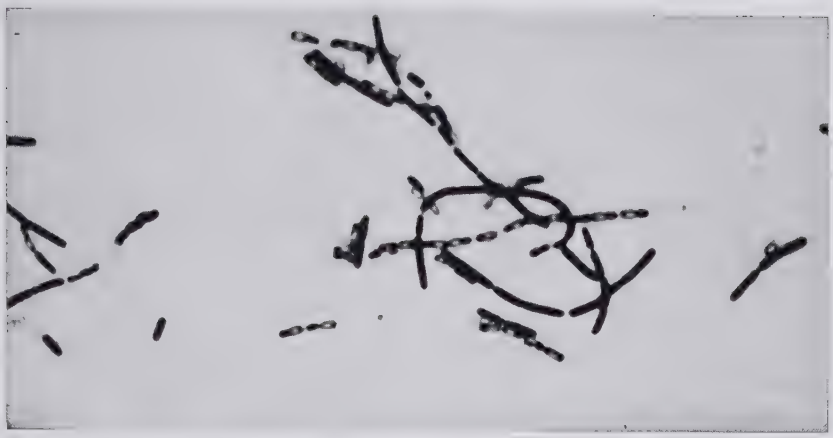


Figure 157. *Bacillus anthracis*, 48-hour culture on nutrient agar. Crystal violet stain. The spores appear as unstained areas. Note the typical arrangement of the bacilli in coiled chains; × 1200.

be the same in both virulent and avirulent strains, and its immunological function is not clear.

The anthrax bacillus also differs from most other aerobic pathogenic bacteria in that it forms spores which are visible as refractile bodies either free or located centrally within the cell. Their diameter does not exceed that of the vegatative cell, and hence the spore-containing rod is not distorted. Spores are formed most abundantly at 32° to 35° C. and only under aerobic conditions, *i.e.*, not in the circulating blood of infected animals. Germination of the spore is usually polar, that is, parallel with the long axis, but may be rarely equatorial.

The bacilli stain readily, but often unevenly, with the usual aniline dyes. The granular material within the cell consists of fat, volutin, or glycogen. The bacillus is gram-positive. The spores are stained with difficulty, but after staining with hot carbol-fuchsin are equally difficult to decolorize; hence the vegatative cells may be decolorized and stained with a contrasting dye.

The colonies of the anthrax bacillus are irregular and have a curled or hairlike structure, giving what is sometimes called a "Medusa head" appearance. On microscopic examination tangled coils of long chains of bacilli may be found. This colonial appearance is closely simulated by *B. subtilis* and some other related saprophytic, aerobic, spore-forming bacilli.

Physiology. The anthrax bacillus grows readily upon all the ordinary laboratory mediums, and growth is not improved by the addition of enriching substances. It can be grown on simple synthetic mediums; thiamin, magnesium, iron, and calcium are required together with a source of energy, and uracil, adenine, guanine, and manganese markedly stimulate growth. Growth occurs at temperatures as high as 41° to 43° C., with an optimum at 37° C. This bacillus is aerobic and facultatively anaerobic. Dextrose and trehalose are fermented rapidly but without gas production. Sucrose, maltose, and some other carbohydrates are fermented less rapidly; lactose, galatose, mannitol, dulcitol, rhamnose, and xylose not at all. Gelatin is slowly liquefied but indol is not formed, nitrate is not reduced, and little or no hydrogen sulfide is produced. Milk is feebly acidified and is curdled by a rennet-like ferment, and the casein slowly peptonized. On potato a gray, furry growth is produced; spores are formed in particular abundance on this medium.

The anthrax bacillus may be differentiated from other species of Bacillus without difficulty except in the case of the saprophytic form, *B. cereus* (see differential key below), and a selective medium for the isolation of spores, which contains propamidine (0.01 per cent) and polymyxin B (20 μg./ml.), inhibits the growth of most nonsporulating aerobic bacilli except Proteus, but does not

inhibit the growth of *B. cereus*.[22] Nevertheless, *B. cereus* is not an avirulent variety of the anthrax bacillus, and the two are at best only distantly related. While animal pathogenicity is the outstanding differential character of *B. anthracis*, it may be identified with a considerable degree of confidence by the aggregation of physiological and morphological characteristics.[9, 18, 28]

The vegetative cells of the anthrax bacillus display the usual degree of resistance to deleterious influences, but the spores are relatively highly resistant, although not so resistant as the spores of *B. subtilis* and related forms. Spores exposed to daylight at room temperatures have been found to germinate after 22 years but not after 22½ years. Anthrax spores are usually destroyed by boiling for 10 minutes and by dry heat at 140° C. for 3 hours. Their resistance to disinfectants is variable, for 0.1 per cent mercuric chloride may fail to kill them in 70 hours, while those disinfectants which are oxidizing agents are much more effective; 3 per cent hydrogen peroxide kills in 1 hour, and 4 per cent potassium permanganate in 15 minutes. In the animal carcass vegetative cells are destroyed during anaerobic putrefactive changes in 72 hours, but spores are viable under such circumstances for at least 9 months. In the soil anthrax spores may remain viable for many years.

Variation. The rough variant is the virulent and naturally occurring form of *B. anthracis*. It was early noted by Pasteur that prolonged cultivation of these bacilli at higher than optimum temperatures, 42.5° C., resulted in a loss of virulence and the appearance of asporogenous variants. A number of different types of variants are produced by such cultivation at high temperatures or in the presence of dilute antiseptics. Smooth and mucoid types of colonies have been observed, and some of these variants may be non-spore-forming. Virulence is not related to the ability to form spores, for both asporogenous virulent strains and spore-forming avirulent strains may be produced.

Virulence of the anthrax bacillus is, rather, dependent in part on the formation of the glutamyl polypeptide capsule. The bacilli of virulent R type appear rough in the conventional culture because they are not encapsulated, but they produce capsules *in vivo* and *in vitro* when the cultures are incubated under increased carbon dioxide tension in bicarbonate-containing mediums, and the colonies are mucoid in appearance. Avirulent R variants resemble the virulent form in culture in air, but the colonies remain rough and capsules are not produced on culture under increased carbon dioxide tension.[11, 43] Avirulent strains may also be encapsulated, producing mucoid colonies in air culture, but do not form toxin.

Figure 158. Colonies of *Bacillus anthracis* on nutrient agar. Twenty-four-hour culture. Note the large size and coarse texture suggestive of R variants; × 3.

Toxins. Under ordinary conditions of culture the anthrax bacillus does not produce an exotoxin, nor is the cell substance of the microorganisms toxic. Vaccines prepared with killed bacilli do not produce an effective immunity, and for many years the high virulence of the anthrax bacillus and the toxemia evident in infections were not understood. As pointed out elsewhere (Chap. 9), the discrepancy between the apparent atoxicity of a microorganism and the toxemia evident in the disease produced by it may be accounted for in two ways, *viz.*, toxicity may be produced by the mircoorganism only under the conditions of growth in the tissues of the infected animals, or the toxicity may be of host origin and produced by the infected tissues.[30, 31]

The former appears to hold true in anthrax in that a toxic substance may be isolated from infected tissue. This was first shown by Watson and his co-workers in 1947 who isolated a substance having inflammatory activity from the tissues of infected animals. Subsequently, the biological activity present in plasma, edema fluid, and exudates in infected animals was found to be separable into two fractions by high speed centrifugation, one designated fraction I in the sediment, and the other, fraction II, in the supernatant. Both are mildly toxic, but in combination apparently act synergistically to account for the high toxicity of the unfractionated plasma.[32, 33, 34, 36] This toxicity is also produced *in vitro* in mediums containing large amounts of serum.[14] The serum appears only to facilitate the passage of one fraction of the toxicity through sintered glass filters, and when it is not included the filtrate is atoxic. The adsorbed fraction may be eluted from the filter with alkaline buffer, and the combination of the eluate and filtrate is toxic,[44] *i.e.*, the toxicity formed *in vitro* consists of two fractions neither of which alone is toxic. The fractions, produced *in vivo* or *in vitro*, are immunologically distinct.[27]

The activity of the toxicity is not yet precisely defined,[29] but it appears to be in the nature of an aggressin-like substance, having high antiphagocytic activity, and is consistent with the disease picture of anthrax and the highly invasive character of the microorganism.

Pathogenicity for Lower Animals.[8, 37] In nature anthrax is primarily a disease of cattle and sheep; horses and swine are susceptible but are less commonly affected. In the period 1945 to 1952, 2785 outbreaks of anthrax occurred in which 14,708 domestic animals were lost in thirty-eight states in this country. Wild deer and other gregarious herbivora are liable to occasional outbreaks. The smaller rodents are very sensitive to inoculation.[19] Rabbits, guinea pigs, and white mice are susceptible in that order, and are fatally affected by the subcutaneous introduction of a very small number of virulent bacilli. The guinea pig is often used as an experimental animal.[26] The white mouse may succumb to inoculation with a single bacillus of a highly virulent strain. Carnivorous animals, though possessing greater resistance than the herbivora, are nevertheless susceptible, as several epidemics in zoological gardens involving leopards, lions, pumas, bears, and other animals have shown. Certain animals possess a marked natural resistance to anthrax. Rats are quite resistant,[41, 42] especially the white rat, only about 14 per cent of the latter dying as a result of inoculation The mature dog is only slightly susceptible. Birds, especially pigeons, can be infected, but not easily. Frogs are completely resistant, but toads are very susceptible.

The route by which the bacilli enter the body exerts an important influence in both experimental and natural infections. Subcutaneous inoculation is the method most commonly practiced in experimental work and is almost uniformly fatal with the ordinary small laboratory animals. Feeding experiments show that administration of spore-free cultures even to highly susceptible animals is without result, owing to the destruction of the bacilli in the stomach. The feeding of spores, on the other hand, leads to infection of the more susceptible species, although not so certainly as subcutaneous

inoculation; resistant species, such as swine, may be infected through the alimentary tract only with difficulty. Infection through the respiratory tract is possible in the experimental animal[2] but is probably almost unknown in the lower animals under natural conditions.

In highly susceptible animals the disease is acute and runs a rapid course; the case fatality in cattle and sheep is about 80 per cent. It presents all the characteristics of typical septicemia, and local manifestations may be almost entirely absent. Enormous multiplication of the bacteria takes place in the blood and internal organs, and sections through the liver or spleen show the capillaries gorged with masses of bacteria. The spleen is a deep red color and greatly enlarged, hence the name splenic fever. The more resistant animal species do not develop this generalized infection, but the bacteria remain localized in an abscess or carbuncle and fail to spread through the body. This is the case with the dog and in some forms of infection in man. Man stands perhaps midway in susceptibilty between the dog and the sheep.

Under natural conditions cattle and sheep are infected through the alimentary tract by swallowing spores while grazing in infected pastures. As has been pointed out, spores are able to retain their vitality in soil for a long period, and pastures once infected may infect cattle after a lapse of as many as 30 years. In this country contaminated feed, especially bone meal, has been responsible for the infection of livestock with anthrax. Hides imported from China and other countries where the disease prevails are not uncommonly contaminated with anthrax spores; in the United States several outbreaks of anthrax among cattle with some consequent cases of human infection have been traced to the overflowing of pasture land by streams receiving the drainage of tanneries.

Cattle may also occasionally be infected by direct contact through wounds, abrasions, and other injuries to the skin; but alimentary infection is by far the most common. Anthrax has been experimentally transmitted to susceptible animals by biting flies of various species that had previously fed on animals dying from anthrax. The bacilli persist in the insects for only a short time.

Pathogenicity for Man.[13] Three routes of infection of human beings are known: (a) through the skin, (b) through the respiratory tract, and (c) through the alimentary tract. The bacillus is almost always transmitted to man through the agency of the lower animals rather than through other human beings. The persons most commonly affected are those having to do with cattle and their products, such as butchers, shepherds, and herdsmen, handlers of hides, hair, and fleeces.[16, 21, 47] In the United States there was a total of 400 cases of anthrax in the period 1945 to 1955. The incidence of cases of infection from wool and hair increased nearly fivefold in the second 5-year period, and over 90 per cent of the infections contracted in tanneries were from goatskins. In the period 1956–1960, 115 cases of anthrax were reported in this country.

During the First World War less efficient preliminary disinfection of hides and bristles permitted the introduction of anthrax-contaminated articles from parts of Asia and South America, and a striking increase in anthrax occurred from the use of shaving brushes—the bacilli were isolated from brushes purchased in the open market in some instances. The bacilli are destroyed on such brushes by soaking in 10 per cent formalin at 110° F. for 4 hours. Laboratory infections, sometimes fatal, have been known to occur with pure cultures of the anthrax bacillus. The case fatality of untreated anthrax in man is probably about 20 per cent.

Cutaneous anthrax (malignant pustule). The most common form of anthrax in the human subject is due to skin infection and usually takes the form of a localized boil or abscess, which often heals spontaneously but may progress into a septicemic condition unless checked by incision or other surgical procedure. Owing to the relatively high resistance of man septicemia does not often

occur, especially if the carbuncle is incised and thoroughly drained. Lesions of all sizes may be produced, from a minute pustule to a large abscess.

Pulmonary anthrax.[1] The pulmonary form of anthrax due to inhalation of the microorganisms is the most dangerous, although not the most common, variety of the disease in man. It is an occupational disease among those who handle and sort wools and fleeces and contract the infection by inhalation of spores set floating in the air from the infected material; pulmonary anthrax is known in England as "wool sorters' disease." It is characterized by many of the symptoms of pneumonia and often passes into fatal septicemia. In experimental airborne anthrax a very few spores suffice in the case of virulent strains, entering the tissues from the alveoli via the lymphatic system. The inhaled spores produce only slight local reaction except for clogging of the capillaries in the terminal stage of the disease. It is of some interest that anthrax spores may occur in the nose and throat of healthy persons exposed to inhalation infection without subsequent development of the disease,[10] and serological survey following an outbreak of pulmonary anthrax suggested the occurrence of subclinical infection.[7, 23] There seems to be no evidence that such persons play any significant part in dissemination of the human infection, and the presence of the microorganisms probably represents no more than a transient contamination. Such observations suggest, of course, that man has appreciable resistance to inhalation infection.

Intestinal anthrax. The alimentary tract, although the usual path of infection in cattle, is very rarely so in man. A few instances are on record of intestinal anthrax contracted through the medium of spore-infected food. Such cases occur among workers with animal products and have probably been due to lack of caution in handling food with uncleansed hands. Insufficiently cooked meat from anthrax-infected animals may also be a source of intestinal anthrax.

Bacteriological Diagnosis. If the specimen is fresh and not grossly contaminated, the anthrax bacillus may be found in gram-stained smears as a rule and is readily cultured on the usual nutrient mediums. Such a specimen may also be used for guinea pig inoculation, but it must be borne in mind that the sporulating obligate anaerobes will kill as rapidly as the anthrax bacillus. In any case the isolated culture must be tested for pathogenicity by animal inoculation. The animal will die in 36 to 48 hours after subcutaneous inoculation of a very small amount of culture. A gelatinous infiltration will be found at the site of inoculation, and the tissues of the animal contain enormous numbers of the bacilli; smears of cut spleen, for example, will show many of the large, gram-positive bacilli. This demonstration of pathogenicity is sufficient for identification since none of the aerobic sporulating rods that resemble the anthrax bacillus are pathogenic for the guinea pig and similar experimental animals.

A precipitation test, the Ascoli test or thermoprecipitation test, is sometimes used to detect anthrax contamination of hides or other tissues. The specimen is extracted with boiling water and the extract used as an antigen in a precipitin ring test with very high titer anthrax antiserum.

Chemotherapy. Prior to the development of the sulfonamides and antibiotics, anthrax was treated with only partial success by a combination of antiserum and arsenicals. While the number of cases is limited by the low incidence of the disease, it has been found that sulfonamides are effective chemotherapeutic agents, and penicillin remarkably so. In those instances in which the bacilli have been found to be penicillin-resistant, the tetracyclines have been found to be practically equally effective.

Immunity.[40] Natural immunity, or resistance, of certain animal species is very high. This is a consequence, at least in part of an anthracidal activity present in tissues and associated with a histone-like protein or polypeptide (Chap. 9).

Recovery from infection in the susceptible animal results in a solid immunity and the serum contains protective anti-

body to high titer. Nevertheless, it has not been possible to produce any appreciable immunity to challenge inoculation by inoculation with killed vaccines of anthrax bacilli, and an effective immunity has resulted only from infection with attenuated strains as used by Pasteur in his early studies on immunity to anthrax in sheep, or by inoculation with virulent, or partially virulent, strains in conjunction with protective antiserum.

With examination of the immunological activity of the products of *in vivo* growth of anthrax bacilli, as noted above in connection with the toxin, it was found by Cromartie and his colleagues that antigen eliciting an effective immune response was produced *in vivo,* but not ordinarily in culture *in vitro.* In 1946 Gladstone found that the immunizing antigen could be produced *in vitro* culture in the presence of body fluids containing albumin and a dialyzable fraction replaceable by sodium carbonate. The elaboration of this antigen in culture and its nature have subsequently been studied at length.[5, 38]

The immunizing antigen is present in cell-free filtrates of *in vivo* or *in vitro* cultures, and apparently diffuses freely from the bacterial cells. It is heat-labile, somewhat unstable but can be preserved by lyophilization, and is protein in nature. It is formed in culture in chemically defined but complex mediums containing many amino acids, purines, glutamine, thiamin, bicarbonate and other salts.[49] Certain of the amino acids, bicarbonate and calcium are required for formation of the antigen but not for growth.[24] It may be precipitated by alum,[48] or salted out and purified by adsorption and elution.[39] The activity is present in maximal amount in young cultures, and there is reason to believe that it tends to be destroyed by bacterial polypeptidases.

This immunizing antigen is not related to the glutamyl polypeptide capsular substance, although both are required for virulence, and the antigen is produced by avirulent nonencapsulated mutants. It is intimately related to the toxin described above, but the relationship has not yet been precisely defined.

While an effective immunity is produced by prophylactic inoculation with the immunizing antigen, in man as well as monkeys and other experimental animals, the nature of the immunity so produced is not completely clear. It presumably includes antibody to the aggressin-like antiphagocytic activity and is antitoxic in that the skin lesion produced by intradermal inoculation in the rabbit is inhibited by immunization; this inhibition has been proposed as a measure of immunity to the infection.[4]

Prophylactic immunization. Active immunization of man to anthrax is not quantitatively significant under ordinary circumstances, but effective immuniza-

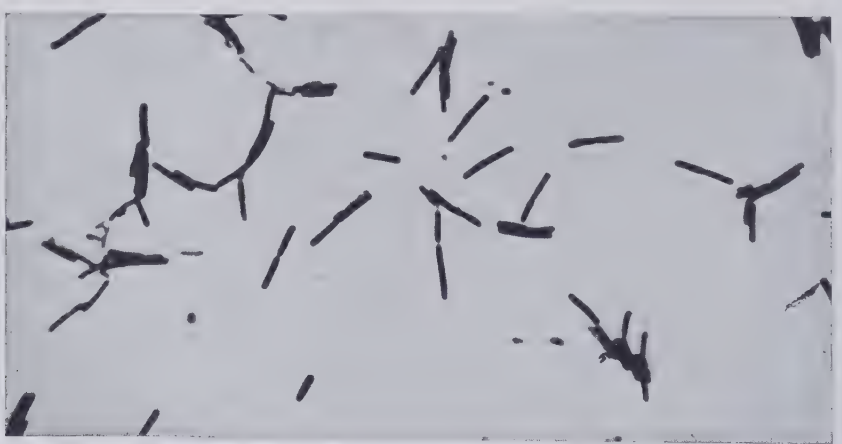


Figure 159. *Bacillus subtilis,* 24-hour culture on nutrient agar. Crystal violet stain. No spores have formed as yet. Note the typical arrangement of the bacilli; × 1200.

Figure 160. Colonies of *Bacillus subtilis* on nutrient agar. Twenty-four-hour culture. Note the resemblance to colonies of the anthrax bacillus; × 3.

tion of cattle, sheep, and other domestic animals can be of very considerable importance.

Immunization by inoculation with attenuated, partially avirulent bacilli, Pasteur's prophylactic, has been widely used in France for the immunization of domestic animals although infection, "vaccine anthrax," may occur. An alternative method, and one which has been used in this country in areas where anthrax is prevalent, is Soberheim's method. It consists of the simultaneous inoculation of virulent spore suspension and immune serum from recovered animals. Either method tends to perpetuate anthrax infection.

The possibility that an effective immunity may be elicited by inoculation with sterile, cell-free preparations of the immunizing, or protective, antigen described above is of considerable practical importance. While it is fully established that an effective immunity is so produced,[3, 6, 45] in the absence of some method of quantitating the immunity, the efficacy of the antigen is difficult to evaluate. The intradermal reaction noted above, and correlation of protection with complement-fixing antibody titer,[20] have been suggested, but an immunity index has been devised[12] which appears to be more informative. On the basis of the last, it has been found[17] that a nonencapsulated avirulent mutant used as a vaccine gave a 10-15-fold increase in immunity, the protective antigen a 1000-fold increase, but immunization with protective antigen followed by inoculation with live vaccine gave as much as 10^9-fold increase over that of uninoculated animals. Such immunization procedures have not yet been tested on a large scale under natural conditions.

RELATED BACILLI

As indicated earlier, there are many species of aerobic sporulating bacilli closely related to and indistinguishable from the anthrax bacillus on any basis other than pathogenicity. The close morphological similarity led many of the early workers to describe "avirulent anthrax bacilli," "pseudo-anthrax bacilli," and "Bacillus anthracoides." There is, however, little basis for such differentiation. The vast majority of these bacilli are saprophytic soil forms and are commonly found as contaminants on plates because of the wide distribution of their spores in dust.

Of the commonly encountered forms, *B. subtilis*, *B. megaterium*, and *B. cereus* are among the most familiar. *B. mycoides*, sometimes called *B. ramosus*, is closely related to *B. cereus* and is classified as a variety of it rather than a separate species. There are some thirty species of Bacillus in all. The following per-

Figure 161. Colonies of *Bacillus mycoides* on nutrient agar. Twenty-four-hour culture; × 3.

tion of a key to the genus indicates the manner in which species are separated and defined. The close relation of the anthrax bacillus to the saprophytic forms is noteworthy.

Mesophilic, aerobic bacilli with spores ellipsoidal to cylindrical and central to terminal:
- (I) diameter of vegetative cells less than 0.9 μ (small cell variety)
  - (1) grow at pH 6.0; acetyl-methylcarbinol formed
    - (a) gelatin hydrolyzed
      - (i) starch hydrolyzed; nitrate reduced to nitrite
        Bacillus subtilis
      - (ii) starch not hydrolyzed; nitrate reduced to nitrite
        Bacillus pumilus
    - (b) gelatin not hydrolyzed
      Bacillus coagulans
  - (2) no growth at pH 6.0; acetyl-methylcarbinol not formed
    - (a) casein digested; urease not formed
      Bacillus firmus
    - (b) casein not digested; urease formed
      Bacillus lentus
- (II) diameter of vegetative cells 0.9 μ or more (large cell variety)
  - (1) acetyl-methylcarbinol not produced
    Bacillus megaterium
  - (2) acetyl-methylcarbinol produced
    - (a) saprophytic, usually motile
      Bacillus cereus
      Bacillus cereus var. *mycoides*
    - (b) pathogenic, nonmotile
      Bacillus anthracis

These forms can be distinguished from one another on the basis of details of spore formation, differential fermentations, and the like, and constitute stable types. Immunological investigation has confirmed the homogeneity of these types also. The immunological specificity of the spores appears to be different from that of the cell substance, and four main types of the small cell group can be distinguished though differentiation of the large cell group by this means is not so satisfactory. Similar results have been obtained in studies of the specificity of the cell substance.

The pathogenicity of these forms is very slight at best, but *B. subtilis* is occasionally responsible for infection,[15] particularly of the eye, and rarely may produce septicemia in the immature animal. Other bacteria of this group are occasionally found to have feeble pathogenic powers. Heaslip[46] isolated an aerobic sporulating bacillus which he called *B. tropicus* by the inoculation of mice with blood from persons suffering from a mild infection in Australia called "coastal fever." This bacillus has also been found there as a natural parasite of the rat and the bandicoot. It appears to be very similar to a bacillus described by Scott many years before as *B. seroficus. B. alvei,* the cause of foul brood of bees, is not pathogenic for man.

REFERENCES

1. Albrink, W. S.: Pathogenesis of inhalation anthrax. Bact. Rev., 1961, **25:**268–273.
2. Albrink, W. S., and Goodlow, R. J.: Experimental inhalation anthrax in the chimpanzee. Amer. J. Path., 1959, **35:**1055–1065.

3. Auerbach, S., and Wright, G. G.: Studies on immunity to anthrax. VI. Immunizing activity of protective antigen against various strains of *Bacillus anthracis*. J. Immunol., 1955, **75:** 129–133.
4. Belton, F. C., and Henderson, D. W.: A method for assaying anthrax immunising antigen and antibody. Brit. J. Exptl. Path., 1956, **37:**156–160.
5. Belton, F. C., and Strange, R. E.: Studies on protective antigen produced in vitro from *Bacillus anthracis:* Medium and methods of production. Brit. J. Exptl. Path., 1954, **35:**144–152.
6. Boor, A. K.: An antigen prepared in vitro effective for immunization against anthrax. I. Preparation and evaluation of the crude protective antigen. J. Infect. Dis., 1955, **97:**194–202.
7. Brachman, P. S., Plotkin, S. A., Bumford, F. H., and Atchison, M. M.: An epidemic of inhalation anthrax: The first in the twentieth century. II. Epidemiology. Amer. J. Hyg., 1960, **72:**6–23.
8. Bulletin: Anthrax. U. S. Department of Agriculture, Farmer's Bulletin No. 1736. 1955.
9. Burdon, K. L., and Wende, R. D.: On the differentiation of anthrax bacilli from *Bacillus cereus*. J. Infect. Dis., 1960, **107:**224–234.
10. Carr, E. A., Jr., and Rew, R. R.: Recovery of *Bacillus anthracis* from the nose and throat of apparently healthy workers. J. Infect. Dis., 1957, **100:**169–171.
11. Chu, H. P.: Variation of *Bacillus anthracis* with special reference to the non-encapsulated avirulent variant. J. Hyg., 1952, **50:**433–444.
12. DeArmon, I. A., Jr. et al.: Immunological studies of anthrax. I. An index to determine quantitative immunization. J. Immunol., 1961, **87:**233–239.
13. Gold, H.: Anthrax. A report of one hundred seventeen cases. Arch. Intern. Med., 1955, **96:**387–396.
14. Harris-Smith, P. W., Smith, H., and Keppie, J.: Production in vitro of the toxin of *Bacillus anthracis* previously recognized in vivo. J. Gen. Microbiol., 1958, **19:**91–103.
15. Heaslip, W. G.: *Bacillus tropicus,* a new species isolated from man and animals described and compared with other bacilli resembling *Bacillus anthracis*. Med. J. Australia, 1941, **2:**536–540.
16. Kendall, C. E.: Occupational anthrax in the United States. J. Occupational Med., 1959, **1:** 174–177.
17. Klein, F., et al.: Immunological studies of anthrax. II. Levels of immunity against *Bacillus anthracis* obtained with protective antigen and live vaccine. J. Immunol., 1962, **88:**15–19.
18. Leise, J. M., Carter, C. H., Friedlander, H., and Freed, S. W.: Criteria for the identification of *Bacillus anthracis*. J. Bact., 1959, **77:**655–660.
19. Marchette, N. J., Lundgren, D. L., and Smart, K.: Intracutaneous anthrax infection in wild rodents. J. Infect. Dis., 1957, **101:**148–153.
20. McGann, V. G., Stearman, R. L., and Wright, G. G.: Studies on immunity in anthrax. VIII. Relationship of complement-fixing activity to protective activity of culture filtrates. J. Immunol., 1961, **86:**458–464.
21. Mohr, W.: Der Milzbrand in Deutschland im Laufe der letzten Jahre unter Berücksichtigung der im Hamburger Tropenkrankenhaus beobachteten Fälle. Landarzt., 1960, **36:**9–11.
22. Morris, E. J.: A selective medium for *Bacillus anthracis*. J. Gen. Microbiol., 1955, **13:**456–460.
23. Norman, P. S., et al.: Serologic testing for anthrax antibodies in workers in a goat hair processing mill. Amer. J. Hyg., 1960, **72:**32–37.
24. Puziss, M., and Wright, G. G.: Studies on immunity in anthrax. IV. Factors influencing elaboration of the protective antigen of *Bacillus anthracis* in chemically defined media. J. Bact., 1954, **68:**474–482.
25. Record, B. R., and Wallis, R. G.: Physiochemical examination of polyglutamic acid from *Bacillus anthracis* grown in vivo. Biochem. J., 1956, **63:**443–447.
26. Ross, J. M.: On the histopathology of experimental anthrax in the guinea-pig. Brit. J. Exptl. Path., 1955, **36:**336–339.
27. Sargeant, K., Stanley, J. L., and Smith, H.: The serological relationship between purified preparations of factors I and II of the anthrax toxin produced in vivo and in vitro. J. Gen. Microbiol., 1960, **22:**219–228.
28. Siedel, G.: Ein Versuch zur Differenzierung von *B. cereus* oder *B. anthracis* innerhalb eines Labortages. Zent. f. Bakt., I Abt. Orig., 1959, **175:**382–385.
29. Slein, M. W., and Logan, G. F., Jr.: Mechanism of action of the toxin of *Bacillus anthracis*. I. Effect in vivo on some blood serum components. J. Bact., 1960, **80:**77–85.
30. Smith, H.: The use of bacteria grown in vivo for studies on the basis of their pathogenicity. Ann. Rev. Microbiol., 1958, **12:**77–102.
31. Smith, H.: Studies on organisms grown in vivo to reveal the bases of microbial pathogenicity. Ann. N. Y. Acad. Sci., 1960, **88:**1213–1226.
32. Smith, H., et al.: The chemical basis of virulence of *Bacillus anthracis*. VII. Two components of the anthrax toxin: Their relationship to known immunizing aggressins. Brit. J. Exptl. Path., 1956, **37:**263–271.
33. Smith, H., and Keppie, J.: Studies on the chemical basis of the pathogenicity of *Bacillus anthracis* using organisms grown in vivo. Society for General Microbiology, Symposium No. 5, pp. 126–151. Cambridge University Press, 1955.
34. Smith, H., Keppie, J., and Stanley, J. L.: The chemical basis of the virulence of *Bacillus anthracis*. V. The specific toxin produced by *B. anthracis* in vivo. Brit. J. Exptl. Path., 1955, **36:**460–472.
35. Smith, H., and Zwartouw, H. T.: The polysaccharide from *Bacillus anthracis* grown in vivo. Biochem. J., 1956, **63:**447–454.
36. Stanley, J. L., Sargeant, K., and Smith, H.: Purification of factors I and II of the anthrax toxin produced in vivo. J. Gen. Microbiol., 1960, **22:**206–218.
37. Steele, J. H., and Helvig, R.: Anthrax in the United States. Pub. Health Repts., 1953, **68:** 616–623.
38. Strange, R. E., and Belton, F. C.: Studies on a protective antigen produced in vitro from *Bacillus anthracis:* Purification and chemistry of the antigen. Brit. J. Exptl. Path., 1954, **35:** 153–165.
39. Strange, R. E., and Thorne, C. B.: Further purification studies on the protective antigen of *Bacillus anthracis* produced in vitro. J. Bact., 1958, **76:**192–202.
40. Symposium: Symposium on recent develop-

ments in immunization against bacterial disease. The basis of immunity to anthrax. Proc. Roy. Soc. Med., 1958, **51:**375–384.

41. Taylor, M. J., Kennedy, G. H., and Blundell, G. P.: Experimental anthrax in the rat. I. The rapid increase of natural resistance observed in young hosts. Amer. J. Path., 1961, **38:**469–480.
42. Taylor, M. J., Rooney, J. R., and Blundell, G. P.: Experimental anthrax in the rat. II. The relative lack of natural resistance in germ-free (Lobund) hosts. Amer. J. Path., 1961, **38:** 625–638.
43. Thorne, C. B., Gomez, C. G., and Housewright, R. D.: Synthesis of glutamic acid and glutamyl polypeptide by *Bacillus anthracis.* II. The effect of carbon dioxide on peptide production in solid media. J. Bact., 1952, **63:**363–368.
44. Thorne, C. B., Molnar, D. M., and Strange, R. E.: Production of toxin in vitro by *Bacillus anthracis* and its separation into two components. J. Bact., 1960, **79:**450–455.
45. Tresselt, H. B., and Boor, A. K.: An antigen prepared in vitro effective for immunization against anthrax. III. Immunization of monkeys against anthrax. J. Infect. Dis., 1955, **97:** 207–210.
46. Weinstein, L., and Colburn, C. G.: *Bacillus subtilis* meningitis and bacteremia. Report of a case and review of the literature on "subtilis" infections in man. Arch. Intern. Med., 1950, **86:**585–594.
47. Wolff, A. H., and Heimann, H.: Industrial anthrax in the United States. An epidemiologic study. Amer. J. Hyg., 1951, **53:**80–109.
48. Wright, G. G., Green, T. W., and Kanode, R. G., Jr.: Studies on immunity in anthrax. V. Immunizing activity of alum-precipitated protective antigen. J. Immunol., 1954, **73:**387–391.
49. Wright, G. G., Hedberg, M. A., and Slein, J.: Studies on immunity in anthrax. III. Elaboration of protective antigen in a chemically-defined, non-protein medium. J. Immunol., 1954, **72:**263–269.
50. Zwartouw, H. T., and Smith, H.: Polyglutamic acid from *Bacillus anthracis* grown in vivo: Structure and aggressin activity. Biochem. J., 1956, **63:**437–442.

Chapter 29

CLOSTRIDIUM—THE SPORE-FORMING ANAEROBES

THE GROUP of anaerobic sporulating bacilli includes a variety of forms.[52,77] Some of these, the anaerobic nitrogen-fixing bacteria, the butyl alcohol and acetone-producing forms and others, have been discussed earlier (Chap. 5). Still others, however, are pathogenic for man and lower animals, and the more important of these forms—*Clostridium tetani, Cl. septicum, Cl. welchii, Cl. novyi, Cl. histolyticum, Cl. chauvoei, Cl. botulinum,* and the nonpathogenic but common species *Cl. sporogenes*—will be considered here.

The status of these bacteria as parasites is open to some question. They occure in the soil, in particular abundance in manured soils, and are found in the intestinal tract of man and animals. *Cl. welchii,* for example, is uniformly present in human feces, and the tetanus bacillus is often found (in up to 40 per cent of specimens examined) in the feces of domestic animals. It has been assumed by some that these bacilli are parasitic and their presence in the soil is a consequence of contamination. Although their numbers are unquestionably greatly increased by manuring and other forms of contamination, some have been found in virgin soils. It is perhaps best to regard them as essentially saprophytic soil forms that are capable of maintaining themselves in the large intestine.

None of these bacteria possesses any marked ability to invade the body tissues by itself. *Cl. botulinum* is apparently incapable of setting up an infection, while others, such as the tetanus bacillus, produce local infections when aided by traumatic injury to the tissues and frequently by the presence of other bacteria. Still others, such as the bacilli associated with gas gangrene, show pronounced invasive properties when once established, but the initial invasion is made possible by other factors, usually trauma and the presence of other bacteria.

The pathogenicity of the anaerobic bacilli is, rather, attributable to their ability to form powerful exotoxins, a property which is curiously confined to these bacteria, the diphtheria bacillus, and the Shiga dysentery bacillus. In the case of botulism the toxin is preformed outside the animal body and, since it is unique in that it is resistant to the digestive enzymes, enters the body by way of the alimentary tract and absorption into the tissues. In the other cases a focus of infection is established, and the toxin formed at that point is disseminated through the body. In some instances, such as gangrene, an extensive local destruction of tissue occurs, but in general the diseases caused by these bacilli are essentially toxemias.

It will be clear from the foregoing considerations that infections with the sporu-

Morphological and Biochemical Characteristics of the More Important Pathogenic Anaerobes

	SPORES	CAPSULE	MOTILITY	PROTEOLYSIS	FERMENTATIONS			
					Dextrose	Lactose	Sucrose	Exotoxin
Cl. tetani	spherical, terminal	−	+	−	−	−	−	+++‡
Cl. septicum	oval, subterminal	−	+	+sl*	+	+	−	++
Cl. welchii		+	−	+sl	+	+	+	++
Cl. novyi		−	+	+sl	+	−	−	+++
Cl. histolyticum		−	+	+	a†	−	−	+
Cl. sporogenes		−	+	+	+	−	−	−
Cl. chauvoei		−	+	+sl	+	+	+	++
Cl. botulinum		−	+	±	+	±	−	+++

* sl: relatively slight; a †:acid; ‡ strong, moderate, and weak.

lating anaerobes are not common under ordinary circumstances, for in most instances traumatic injury is a preliminary to infection. On the battlefield, however, such injuries are common, and tetanus and gangrene are not infrequent complications of war wounds. Such anaerobic wound infections were prominent in the First World War, perhaps as a consequence of battles fought over the heavily manured fields of France. The occurrence of gaseous gangrene during World War II among troops in North Africa where the desert soil is relatively free of such forms suggests that clothing may be a more important source of contamination than had been supposed.

Cl. histolyticum is micro-aerophilic, *i.e.*, it will grow in the presence of small amounts of oxygen, but the remainder of the forms considered here are obligate anaerobes. They may be isolated in pure culture by picking colonies from a shake culture or from plates incubated in an anaerobic jar. All are large gram-positive rods, nonencapsulated with the exception of *Cl. welchii*, and motile by means of peritrichous flagella with the exception of *Cl. welchii*. Spores are usually of a greater diameter than the vegetative cells, and the spore-containing cells are spindle- or club-shaped. The spores of the tetanus bacillus are round and terminal, and those of the other bacilli oval and subterminal. Two general physiological types of anaerobic bacilli may be distinguished, the one predominantly fermentative or saccharolytic and the other predominantly proteolytic. These and other characteristics are summarized in the accompanying table.

Large amounts of volatile organic acids are produced in the fermentation of carbohydrates by these bacilli; they differ in this respect from the other pathogenic bacteria which produce nonvolatile acid, *i.e.*, lactic acid, for the most part. Amino acids are vigorously attacked by the obligate anaerobes; some are "fermented" to organic acids, while others are mutually oxidized and reduced.

CLOSTRIDIUM TETANI

Tetanus is a disease of man and animals characterized by spasms of the voluntary muscles. The spasms are often most marked in the muscles of the jaw and neck, hence the name "lockjaw." Tetanus bacillus was first described in 1884 by Nicolaier, who observed it in the pus taken from mice and other animals that had died after subcutaneous inoculation with small quantities of soil. Kitasato isolated the microorganism in pure culture in 1889 and demonstrated

its causal significance. He also proved the inability of the tetanus bacillus to invade the blood stream and showed the disease to be an intoxication. In 1890 von Behring and Kitasato laid the basis for antitoxic therapy in their discovery of diphtheria and tetanus antitoxins.

Morphology. Individual tetanus bacilli are slender, motile (twenty to thirty peritrichous flagella), gram-positive, sporulating rods with rounded ends. Their common dimensions are 0.3 to 0.5 μ in width and 2 to 5 μ in length, but vegetative filaments of much greater length occur. The shorter forms are usually straight; filaments tend to curve in an undulating manner. Short chains or rods may occur. The spore is spherical and terminal and larger in diameter than the vegetative cell; spore-containing cells have a characteristic drumstick appearance. Isolated colonies in deep dextrose agar have a wooly appearance and may either be flocculent or have an opaque center. Surface colonies are flat, rhizoid, or even feathery, and frequently exceed 1 mm. in diameter. Later the centers may become slightly raised. Colonies on blood agar show hemolysis.

Physiology. The tetanus bacillus develops in plain or dextrose broth and in brain, meat, agar, and gelatin mediums from which the air has been expelled by heating and excluded by some form of seal. If the depth of mediums is adequate, say 7 to 12 cm., no special seal is required, especially for the more viscous mediums. Growth occurs between 14° and 43° C.; the optimum temperature is 37° C.

The growth of *Cl. tetani* is influenced greatly by the presence of associated microorganisms. In sugar-free mediums it may be grown in mixed cultures upon the surface of culture mediums in contact with air through the absorption of oxygen by the associated aerobes. But in dextrose broth the growth of the tetanus bacillus in mixed culture is likely to be inhibited by acid formation due to the associated bacteria. Sugar-free mediums, such as meat infusion peptone broth or deep meat or brain mediums, are therefore preferable for initial culture from contaminated material. Pure cultures may be isolated from these initial cultures by deep agar or surface culture methods. It has been supposed that an initial heating of the contaminated material simplifies isolation of the tetanus bacillus by destroying the vegatative cells of other microorganisms that may be present; this method may, however, leave the spores of the tetanus bacillus still badly mixed with those of other aerobic and anaerobic bacteria, some of which may be more resistant than the tetanus spores.

In pure culture, dextrose stimulates growth as in the case of the other anaer-

Figure 162. *Clostridium tetani* in pure culture. Young, actively growing culture showing beginning spore formation. Note the refractile, unstained spores, the drumstick appearance when these are attached to the cells, and the tendency of the vegetative cells to remain attached end to end. Fuchsin; × 1150.

obes, although dextrose and other carbohydrates are not fermented by *Cl. tetani*. Sporulation is not inhibited by carbohydrates, as with the fermentative anaerobes; on the contrary, sporulation of the tetanus bacillus is accelerated in dextrose broth.

Growth in gelatin stab is slow at 22° C. Incubated at 37° C. for 2 to 3 days, gelatin cultures usually fail to stiffen in the refrigerator. Coagulated proteins, such as blood serum and egg white, are very slowly liquefied. Deep brain and meat mediums may be slightly softened, but never digested fully; after several weeks a slight darkening occurs near the surface exposed to air. The tetanus bacillus is, therefore, only weakly proteolytic. Amino acids are utilized readily but not by paired oxidation-reduction; glutamic acid, aspartic acid, and serine, for example, are attacked directly with the formation of CO_2, NH_3, and acetic and butyric acids.[16] Both amino acids and carbon compounds are readily dehydrogenated. In litmus milk there is reduction of the indicator and sometimes a slight precipitation of the casein. Nitrates are not reduced to nitrites, but hydrogen sulfide and indol are produced.

The tetanus bacillus has been grown in synthetic mediums,[27, 43, 58] and its growth requirements are relatively complex, including the amino acids arginine, histidine, tyrosine, valine, leucine, isoleucine, and tryptophan; the bacterial vitamins riboflavin, pantothenic acid, thiamin, folic acid, biotin, pyridoxine, and nicotinic acid; the purines adenine and uracil; and oleic acid. The formation of toxin is inhibited by iron, and best yields are obtained when as much iron as possible is taken out of the medium, though undoubtedly very small amounts are required. A beef infusion medium containing hog stomach autolysate or tryptic digest of casein, together with glucose, carbonate, potassium, magnesium, and phosphate, allows the production of toxin to high titer.

Spore formation begins in about 2 days at 37° C. and in 8 or 10 days at room temperature. The spores are highly resistant and when protected from light and heat remain viable for years. Theobald Smith found a number of strains that resisted steaming at 100° C. for 40 to 60 minutes. Five per cent phenol is said to destroy tetanus spores in 10 to 12 hours; the addition of 0.5 per cent hydrochloric acid may reduce the time to 2 hours.

Antigenic Structure. The tetanus bacillus is antigenically heterogenous, and a number of types, designated by Roman numerals, have been described. At present ten in all are known. Both somatic and flagellar antigens appear to be involved, the former of a group and the latter of a specific nature. Type VI organisms are not flagellated and lack type-specific antigen, and types II, IV, V, and IX have a common O antigen which results in higher titer cross-reactions among these than among the other types.[34] Types I and III have been found most commonly in the United States, England, and France, type V in China. The toxin formed by these types is immunologically identical.

Tetanus Toxin.[91] As indicated elsewhere (Chap. 9), the potent soluble toxin produced by the tetanus bacillus is of dual nature; *tetanospasmin* is the portion that affects the nervous tissue and *tetanolysin* is a hemolysin. The former is by far the more important; there is no indication that tetanolysin has any significance in connection with the ordinary symptom complex. Liquid cultures are usually toxic; 5×10^{-6} ml. may be fatal to a mouse. Although apparently diffusing freely into the surrounding medium[79] the toxin is also present in the bacterial cells[54] and may be extracted from washed cells with molar sodium chloride.[70]

The toxin is filterable and may be freed from bacteria by filtration through Berkefeld, Chamberland, and similar filters. In aqueous solution the toxin is highly unstable to heat and light and must be stored in a cold dark place. It is apparently protein in nature, or at least has not as yet been separated from protein, and is destroyed by proteolytic enzymes and hence is ineffective when given by mouth. It may be precipitated

with ammonium sulfate and in the dry state retains its potency for a long time. It is an excellent antigen and gives rise to high titer antitoxic serums.

As indicated above, tetanus toxin is one of the most potent poisons known, and its toxicity far exceeds that of alkaloids and other substances generally regarded as highly poisonous. It has been prepared in crystalline form by precipitation with methanol in the cold. These preparations contained 5 to 7.5 $\times$ 10^7 LD_{50} doses for mice per mg. of nitrogen, and tetanus was produced in mice with as little as 0.000013 γ of the crystalline toxin. Usually an incubation period intervenes between inoculation and appearance of symptoms which cannot be reduced to less than 8 hours with the usual toxic filtrate, and beyond that point the incubation period is inversely related to the amount of toxin injected.[66] With crystalline material, doses of as much as 500,000 MLD are possible in the mouse, and these large amounts produce symptoms in 30 minutes and death in an hour.

Tetanus toxin appears to act like strychnine, suppressing all types of synaptic inhibition[11] and the ultimate cause of death is asphyxia from spasm of respiratory muscles. This spasm can be released by the use of muscle relaxants and anesthetic agents. Such symptomatic treatment, in combination with antitoxin, with curare and similar substances, succinylcholine, sedation as with barbiturates, tracheotomy with positive pressure respiration, and the like, has met with some success,[2, 24, 46, 75, 86] but prolonged anesthesia raises unusual problems, *viz.*, bone marrow damage resulting from prolonged nitrous oxide anesthesia, etc.

The dissemination of tetanus toxin in the body. There are two views concerning the pathway by which tetanus toxin reaches the central nervous system. According to the experimental evidence of Meyer and Ransom, tetanus toxin is absorbed by the end organs of the motor nerves and travels to the ganglion cells of the central nervous system along the axis cylinders of the peripheral nerves. The time consumed in this passage represents the larger part of the incubation period. The toxin may circulate for a time in the blood, but the only path to the central nervous system lies along the axis cylinders of the motor nerve tract. A cut nerve takes up the toxin very slowly and a degenerate nerve not at all. Section of the spinal cord prevents the toxin from reaching the brain. Meyer and Ransom believed that the spinal ganglion of the sensory nerve presents a barrier to the advance of the toxin, and that for this reason sensory nerves are unable to conduct the toxin. The remarkable excitation of the motor cells of the spinal cord that is observed in tetanus is not accompanied by characteristic lesions.

Although long generally accepted, this view was challenged by Abel and his co-workers,[1] who proposed the following theory as in accord with their experimental findings: The toxin exhibits both a central and a peripheral action, each of which may be demonstrated independently of the other. The central effect, which is characterized by reflex motor convulsions, is due to the poisoning of the motor nerve cells of the spinal cord, medulla, and pons; the peripheral effect, recognized as the unremitting rigidity of voluntary muscles, results from the fixation of the toxin upon the motor end organs. Following subcutaneous or intramuscular injection the toxin is absorbed by the lymphatics and distributed to the central nervous system by way of the arterial circulation. Normal tonicity of the motor end organs seems to be essential to the development of tetanic rigidity; neurotomy results in an immediate, sharp depression of the tonicity of the myoneural junctions, hence they are not responsive to the influence of tetanus toxin.

Abel's views have not been generally accepted in spite of their support by convincing experimental evidence which cannot be reviewed here. Other experimental data have been presented in support of the theory of the axis cylinder pathway,[5, 6, 18, 89] and it may be regarded as established.

Tetanus toxin possesses a strong affinity for the cells of the central nervous system. On inoculation into the central nervous system it is rapidly fixed; the fixation time is much less than the latent period, suggesting subsequent action on the nerve tissue, particularly of the medulla.[87, 88] A mixture of toxin and brain substance can be injected into an animal without producing any toxic effect, the toxin apparently entering into a firm combination with some ingredient of the nerve substance.[39, 40] Not only the central nerve cells, but to some extent other tissue cells, are able to bind tetanus toxin. Subcutaneous inoculation is less likely to result fatally than direct inoculation into nerve tissues because some of the toxin is bound and prevented from reaching the highly sensitive nerve cells.

Pathogenicity. Tetanus is essentially an intoxication. The bacilli set up a localized infection, and the toxin formed there is disseminated through the body and gives rise to the symptom complex characteristic of the disease. Bacillemia may occur very rarely, however, and has been produced experimentally. The bacilli generally gain entrance to the tissues by means of a deep, dirty wound which may be relatively small, so small sometimes as to escape serious attention. The widespread occurrence of the tetanus bacillus would seem out of harmony with the relative infrequency of tetanus infection but mere introduction of the bacillus into the body is not sufficient to produce the disease; the microorganisms must find favorable conditions for proliferation at the site of penetration. Experimentally, pure cultures of vegetative cells or spores that have been freed from toxin cannot germinate in uninjured tissues, but simultaneous inoculation with common saprophytes or with irritant chemicals, such as calcium salts or lactic acid, enables the bacilli to grow and form toxin. As indicated elsewhere, a sufficiently low oxidation-reduction potential is necessary for the germination of tetanus spores, and it is not unlikely that the potential of normal tissues is too high to allow germination but is reduced by injury.

Tetanus in man.[4] Tetanus continues to be a highly fatal disease, with a case fatality rate of about 60 per cent in this country; it is claimed that symptomatic treatment as described above may reduce the case fatality rate to as little as 20 to 30 per cent. The incidence of the disease has not changed appreciably since 1947, *i.e.*, after prophylactic immunization with toxoid had been well established by military experience, and averages about 0.3 cases and 0.2 deaths per 100,000 population. Prior to 1948 the seasonal incidence reached a peak in late spring and summer, but since then has shown a peak in late summer and early fall. Half or more of the cases are reported from southern and southeastern states, with Florida having the highest rate of 1.5 and Alabama the next with 1.3 cases per 100,000 during the period 1951 to 1954. Tetanus neonatorum (see below) is an important factor in these higher rates; in Florida during this period the non-white neonatal mortality rate was 34.3 deaths per 100,000 live births.

While as late as 1925 postoperative or surgical tetanus accounted for as much as 10 per cent of the cases, this has become relatively rare although outbreaks still occur in modern hospitals. It is most commonly associated with faulty sterilization of dressings, and occasionally results from the use of contaminated catgut. The disease may also be associated with uncommon circumstances; for example, tetanus among drug addicts, due to the use of unsterilized needles, syringes, or contaminated drugs, has been observed with some frequency in Chicago, and in a scattered way in other large cities in the United States.

By far the most common kind of tetanus is that following injury, even apparently minor or trivial injury, frequently puncture wounds which facilitate the growth of the anaerobic bacilli. Of a group of ninety-one cases treated in the Mayo Clinic,[83] twenty-nine were from wounds with nails and wood slivers, six other types of puncture wounds, thirty-four lacerations and abrasions, seventeen with miscellaneous foci of infection, and in five cases there was no physical evi-

dence of a wound. This kind of tetanus tends to occur in rural males, especially children.

Tetanus of the newborn or *tetanus neonatorum* is a consequence of infection of the umbilicus through septic midwifery. It is especially common among the Negroes of the southern states and in other races living under unhygienic conditions.

The tonic spasms characteristic of tetanus usually begin at the site of infection, and the initial symptoms may include headache and stiffness of the neck. The spasms may remain localized in mild infections, but usually they are general and involve the whole somatic muscular system. Postmortem findings are insignificant; other than a moderate congestion, the organs show no pathological changes and the initial lesion may, of course, be inapparent or small.

The incubation period of tetanus is variable and may range from 2 to 50 days. The case fatality is inversely related to the incubation time; it may be as high as 70 to 80 per cent or as low as 15 to 20 per cent. Death, if it occurs, follows relatively soon after the appearance of symptoms; the dictum of Hippocrates, "such persons as are seized with tetanus die within 4 days, or if they pass these they recover," still stands. When the disease has a prolonged incubation period, a less sudden development of symptoms, and consequently more favorable prognosis, it is sometimes termed "chronic."

Lower animals. Tetanus is not a rare affection in the horse, the symptoms and course of the disease being similar to the disease in man. Cattle, sheep, and hogs are less commonly affected. Experimentally, tetanus can be produced in mice and guinea pigs by the inoculation of spores introduced on splinters of wood and also by injection of toxin. The feeding of animals with tetanus bacilli, spores, or toxin is without effect. Tetanus differs from most other infectious diseases in that the diseased animal is not an appreciable factor in the spread of infection. A normal horse may distribute tetanus spores quite as widely and freely as a horse sick with tetanus.

Immunity. As indicated earlier, tetanus toxin is an excellent antigen, and high titer antitoxic serums may be prepared. Horses are good producers of antitoxin and are immunized by toxin-antitoxin mixtures followed by toxin alone. In France toxoid is used for the first injections.

The standardization of antitoxin. The American immunity unit is defined as "ten times the least quantity of antitetanic serum necessary to save the life of a 350 gm. guinea pig for 96 hours against the official test dose of a standard toxin furnished by the Hygienic Laboratory of the Public Health and Marine Hospital Service." The official test dose is about 100 guinea-pig MLD's of a precipitated standard toxin. It may be noted that the L_0 rather than the L_+ end point is determined. Thus the tetanus antitoxic unit has slightly more than ten times the experimental protective power of the diphtheria antitoxic unit. An antitoxic unit strength of 900 per ml. may be attained exceptionally. Practice has been different in other countries, however, and the Permanent Committee on Standardization of the Health Organization of the League of Nations found that 1 German unit, 66 American units, and 3750 French units were equivalent. It has been agreed that the international unit shall be one half of the American unit.[67]

The appearance of multiple zones of precipitation has interfered with the application of the Ramon flocculation method to tetanus toxin, toxoid, and antitoxin. It has been reported that a specific flocculation zone occurs with refined enzyme-treated antitoxin.[47, 55]

The antitoxin deteriorates with time, the most important factor being the temperature at which it is stored. During storage for 1 year in the refrigerator no potency is lost, at room temperature the loss is 7 to 9 per cent, and at 37° C. it is 44 to 47 per cent.

The prophylactic use of antitoxin.[78, 81] In veterinary practice, tetanus antitoxin has been used prophylactically with a high degree of success. Vaillard collected the statistics from 1896 to 1906 of eight veterinary surgeons who inoculated

13,124 animals after operations or accidental wounds without the occurrence of a single case of tetanus. During the same time two veterinary surgeons alone saw 139 cases of tetanus among animals which did not receive the treatment. The figures of Nocard and Labat added to Vaillard's data cover the cases of 16,917 animals receiving prophylactic injections; among them only one horse had tetanus. In this case the antitoxin was given 5 days after the wound and the attack was mild.

It is probable that in many cases tetanus is averted in man by the prophylactic use of antitoxin. Even though the disease is not prevented, the incubation period is delayed, and the disease may be very mild or remain localized. Precise statistical evidence involving comparable series of controls is not available, but the experience of the First World War was that with increased use of tetanus antitoxin there was not only a lower incidence of the disease but an increase in mild and chronic cases. Passive immunization provides antibody in the circulating blood which combines with toxin and renders it harmless. The avidity of the nervous tissue for tetanus toxin is very great, however, and symptoms may appear in spite of the presence of circulating antibody. Passive immunity is transient and repeated injections of antitoxin at 6- to 7-day intervals must be made as long as danger of infection remains.

From time to time attempts are made to passively immunize the newborn by maternal immunization during pregnancy via transplacental antibody. In general this has not proven satisfactory, but the procedure has given encouraging results where neonatal tetanus is common.[74]

The therapeutic use of antitoxin.[65] Tetanus antitoxin appears to have but limited therapeutic value. It is obvious, of course, that symptoms result from damage to the nerve tissue, and the administration of antitoxin will neither repair such damage nor displace the more avid nerve tissue already in combination with toxin. Reports on the therapeutic value of antitoxin are conflicting; some workers contend that intrathecal administration either alone or combined with intramuscular injection has a beneficial effect. The records of Cook County Hospital in Chicago, however, show that the therapeutic use of antitoxin has not reduced the mortality from tetanus there. It has been reported that mortality may be reduced somewhat, from 76 per cent to 49 per cent, by the use of very large amounts of antitoxin.[12]

Active immunization.[29, 69] In recent years some emphasis has been placed upon active immunization against tetanus, particularly by the French workers. In a summary of the results of 12 years' experience with active immunization of horses and men with formol toxoid, Ramon[68] states that in one cavalry unit in which tetanus was endemic more than 50,000 horses were immunized over a 10-year period and tetanus has practically disappeared, and in a million and a half human beings immunized with toxoid no case of tetanus has occurred.

With the beginning of the Second World War, active immunization against tetanus was adopted by the armed services of France, Britain, and the United States. Both fluid and alum-precipitated toxoid are used, in this country the former by the Army and the latter by the Navy, and appear to be equally effective though three doses of fluid toxoid are required as against two doses of alum-precipitated toxoid. No international standard exists for tetanus toxoid; the United States Army specified that the toxin contain at least 10,000 guinea-pig MLD's per ml. and be detoxified with 0.4 per cent formalin; the final preparation must be atoxic for guinea pigs in 5 ml. amounts, and pigs receiving 1 ml. as an immunizing dose must be able to withstand 10 MLD of toxin at the end of 6 weeks. The importance of more than one immunizing inoculation is illustrated by the observation that the mean antitoxin titer after two inoculations was 0.35 IU (International Units), but with a third inoculation 10 months later the mean titer rose to 10 IU and 18 months later was still 0.37 IU.

More precise examination of the immunogenic potency of toxoids has shown that those prepared in different laboratories may vary widely in this respect, giving an immune response in protective units ranging from 0 to 1,149 IU per ml. of serum.[33] An international standard has been defined[32] which should reduce this variability. The guinea pig is used in assaying immunogenic potency, but the use of the mouse has been urged by many.[17]

The response to booster inoculation is extremely rapid in persons giving an anamnestic reaction, so much so that booster inoculation with toxoid may be substituted for prophylactic antitoxin under circumstances indicating prophylaxis. Prophylactic toxoid was given as a standard procedure in American military personnel during World War II, while British personnel received antitoxin; there was no significant difference in the incidence of tetanus between the two services.

Detectable antitoxin and ability to respond to booster inoculation may persist for as long as 15 years, but it is usually recommended that booster inoculation be given at 4-year intervals.[42, 48]

In practice this immunization appears to be highly effective. In the period 1942 to 1945 only twelve cases of tetanus occurred in the United States Army, of which six were in unimmunized persons; four cases occurred in the United States Navy, of which three were in unimmunized persons; in contrast a tetanus rate of about 10 per 100,000 wounded prevailed in the Japanese army and navy which did not practice routine immunization. British experience in the Middle East war zone similarly indicated an effective immune response, though in most cases only two doses were given. The success of active immunization, coupled with the low incidence of untoward reactions (reported to be 1 in 10,000 immunizations with improved toxoid), has suggested its more general application in civil life. In France, where active immunization to tetanus has been of more and earlier interest than elsewhere, it has been made compulsory as with diphtheria immunization. In this country considerable interest has attached to combined tetanus and diphtheria immunization of children.

Chemotherapy. The chemotherapeutic drugs are not an important adjunct in the treatment of tetanus. As pointed out above, the disease is primarily a toxemia, and the symptoms are a consequence of tissue damage by the toxin. Antitoxin is mandatory to neutralize as yet uncombined toxin, but the chemotherapeutic drugs have no antitoxic activity. There is some evidence that penicillin and tetracyclines partially protect against experimental tetanus when used prophylactically, but active or passive immunization is much more effective.

GASEOUS GANGRENE[59, 76]

Gaseous gangrene is a syndrome which often follows dirty, lacerated wounds, especially those involving fractures. It is a characteristic complication of war wounds, and present knowledge of this affection was largely developed during the First World War. But this disease is by no means so rare in civil life as was formerly thought. The increased number of injuries in automobile accidents is responsible for many cases of gangrene. Men injured about railroad tracks, either employees or vagrants, seem particularly prone to develop gaseous gangrene if not properly and promptly treated. Certain forms of peritonitis, appendicitis, intestinal obstruction, puerperal sepsis, and postoperative infections (particularly after laparotomy) are etiologically closely related to it.

The trauma usually, and perhaps necessarily, preceding the development of gangrene results in a local area of tissue anoxia, anaerobic oxidation of carbohy-

drate continues, and the local reducing intensity drops to levels permitting growth of the obligate anaerobes. Under the conditions of increasing acidity, the catheptic enzymes of the muscle tissue are activated, and free amino acids accumulate as a consequence of the proteolysis to provide an adequate nutrient medium for the invading microorganisms.

Gangrene is usually a mixed infection, and both aerobic and anaerobic bacteria may be isolated from a single gangrenous lesion. The aerobic and facultative anaerobic forms, such as streptococci and coliform bacilli, are not primarily concerned in the development of the pathological process but may contribute indirectly to it by facilitating the exhaustion of locally available oxygen. The gangrenous process is, rather, a consequence of the activity of the sporulating obligate anaerobes and the exotoxins produced by them. It is of more than passing interest that many of these toxins are enzymes; in addition to hyaluronidase, lecithinases, collagenases, and others are produced that have hemolytic, necrotizing, and lethal effects upon the host and host tissues.[49] In the fulminating form of gaseous gangrene the muscle tissue becomes filled with gas and with a serosanguineous exudate depending for its character upon the properties of the associated microorganisms.

Of the various species of pathogenic Clostridia, *Cl. welchii* is usually the most frequently found, closely followed by *Cl. novyi* and *Cl. septicum*. The nonpathogen *Cl. sporogenes* is also found in a considerable proportion of cases; its contribution to the pathology of gangrene is uncertain and it seems probable that its common presence is a reflection of its ubiquitous distribution. Various compilations of the incidence of the several species of Clostridium found in gangrene have been made, and these do not differ significantly from one another. The frequency of occurrence of some of the more commonly found species is illustrated in the accompanying table compiled from World War II data of various authors. It is evident from this that the bacteriology of gaseous gangrene is complicated, and while there is in effect a typical form of gaseous gangrene, (*a*) it is not always produced by the same microorganism; (*b*) it is frequently caused by several associated agents; (*c*) it is often the complex result of the combined action of these principal anaerobic bacilli with various other bacteria which play an indeterminate accessory role.

THE VIBRION SEPTIQUE, CLOSTRIDIUM SEPTICUM

In 1877 and 1881 Pasteur, while studying anthrax, produced septicemia in rabbits and guinea pigs by the inoculation of putrid blood from a cow. The affection could be communicated from individual to individual, and a sporulating, motile, rod-shaped anaerobe, considered by him "one of the vibrions of putrefaction" (the actively motile bacilli sometimes appear to be curved), was regarded as the cause of the septicemia and named "vibrion septique."

In 1881 Koch described the pathological effects of a microorganism which he declared identical with the vibrion septique of Pasteur. But this bacterium failed to produce septicemia in guinea pigs, and since its pathogenic effects were limited largely to the site of inoculation, Koch designated it "the bacillus of malignant edema."

Neither Pasteur's nor Koch's description would suffice now to identify with certainty the microorganisms in question. Fortunately, the original strain of Pasteur's vibrion septique has been maintained in France, so that its outstanding characteristics are well known. The lack of any such legacy from Koch in Germany, due to his failure to recover cultures, has led to an all but interminable discussion as to the properties of the true bacillus of malignant edema. The vibrion septique is now generally known as *Cl. septicum*.

Morphology. The vibrion septique is a gram-positive, sporulating, spindle-shaped rod, or filament, and in young

Incidence of Clostridium Species in Gangrene

ORGANISM	PER CENT OF CASES			
	1	2	3	4
Cl. welchii (*perfringens*)	56	83	80	39
Cl. novyi (*oedematiens*)	37	47	48	32
Cl. septicum	19	24	4	—
Cl. histolyticum	6	6	—	—
Cl. tetani	13	—	8	4
Cl. bifermentens (*sordellii*)	4	35	20	54
Cl. sporogenes	37	50	72	54
Cl. tertium	30	59	8	3

1. MacLennan: Lancet, 1943, i:63, 94, 123 (146 cases).
2. *Ibid.*, 1944, 2:203 (17 cases).
3. Stock: Med. Bull. E. T. O., 1944, 2:159 (25 cases).
4. Smith and George: J. Bact., 1946, 51: 271 (110 cases).

cultures motile, with many peritrichous flagella. The ends are slightly rounded and the spores, which are oval, are usually median and swell the vegetative cell into a clostridium previous to their release. Spores are formed only in mediums not containing fermentable carbohydrate in excess. The long chains and filaments of these microorganisms which occur on the visceral surfaces of infected guinea pigs are of high differential value. Capsules have never been observed. Deep colonies in 1 per cent agar are transparent or semitransparent. Hemolysis occurs on blood agar.

Physiology. *Cl. septicum* is a strict anaerobe and develops readily in deep brain or tissue mediums, producing gas rather abundantly. These mediums are not discolored even in the presence of metallic iron. Gelatin is liquefied, but coagulated serum and other proteins are not digested or blackened. Hydrogen sulfide is produced but indol is not. Dextrose, levulose, galactose, maltose, lactose, and salicin are fermented; mediums not containing one of these sugars support only slight growth. Sucrose, inulin, mannitol, and dulcitol are not fermented. The fermentation of salicin and nonfermentation of sucrose allow the biochemical differentiation of *Cl. septicum* and *Cl. chauvoei*, for the latter does not ferment salicin but does ferment sucrose.

Antigenic Structure and Toxin. Strains of *Cl. septicum* are immunologically related but distinct. Six groups have been distinguished on the basis of two O and five H antigens.[57] These bacilli are immunologically related to *Cl. chauvoei*. The toxin formed, however, is specific, but a relatively weak lethal agent. The MLD for mice is about 0.005 ml. Bernheimer[8] has prepared a dialyzable medium containing casein hydrolysate, cystine, tryptophan, glutamine, biotin, thiamin, nicotinic acid, pyridoxine, glucose, thioglycollic acid, and inorganic salts which supports the growth of some strains with the formation of 400 to 500 mouse LD_{50} doses of

Figure 163. *Clostridium septicum* from pure culture. The tendency to form elongated vegetative cells is apparent. Fuchsin; × 1050.

toxin per ml. When injected into animals it produces a gelatinous edema and some local necrosis of the tissues. The toxin has a specific cardiac action in the cat and rabbit, producing a fall in systemic and a rise in venous blood pressure, in the cat a specific constriction in the pulmonary and coronary circulations, with edema of the lungs and loss of fluid from the circulation. This lethal necrotic toxin, or α-toxin, is an oxygen-labile hemolysin. The β-toxin is a deoxyribonuclease which attacks the nuclei of rabbit leucocytes. Hyaluronidase is formed also, and is sometimes referred to as the γ-toxin.[56]

Pathogenicity. *Cl. septicum* does not occur in gaseous gangrene of man as frequently as some of the other anaerobic bacilli but has been found both alone and in mixed cultures. It has been recovered from gaseous infections in cattle and may be one of several microorganisms responsible for blackleg, usually considered a specific disease due to *Cl. chauvoei.* It has also been found in gaseous infections of hogs and other domestic animals. Experimentally the vibrion septique is strikingly pathogenic for chickens, pigeons, rabbits, guinea pigs, rats, and mice. In such animals the bacteria develop rapidly, producing gas and a reddish, serous edema. They invade the adjacent tissues and the circulation, producing septicemia which is usually fatal within 24 to 48 hours; sublethal doses do not produce any reaction. Impression smears from the tissues, and especially from the liver, usually show elongated filaments or chains as contrasted with the single bacilli found in animals killed with *Cl. chauvoei.*

Antitoxic serums which are prophylactic and, to some degree, curative may be prepared by injection of *Cl. septicum* toxin into horses. The antiserums do not have the high antitoxin content that is found in antitetanic serums. Polyvalent commercial serums for prophylactic and therapeutic use in wound infections often contain septicum antitoxin.

CLOSTRIDIUM WELCHII (CLOSTRIDIUM PERFRINGENS)

Cl. welchii was first cultivated by Achalme in 1891 and supposed by him to be the cause of articular rheumatism. In 1892 Welch and Nuttall isolated this bacillus from the foamy organs of a cadaver and called it *Bacillus aerogenes capsulatus.* Found by Fränkel the following year, it was designated *B. phlegmonis emphysematosae,* and in 1897 Veillon and Zuber called it *B. perfringens.* Sometimes called Fränkel's bacillus in Germany, *Cl. perfringens* in France, and *Cl. welchii* in English-speaking countries, it is designated *Cl. perfringens* by Bergey.

Figure 164. *Clostridium welchii* from pure culture. Note the relatively smaller size of these bacteria and the central spores. Fuchsin; × 1050.

Morphology. *Cl. welchii* is a plump, nonmotile, gram-positive rod of variable length, occurring in chains and singly. Capsules are usually present in preparations made from the organs or body fluids. Spores are formed sparingly and only in the absence of fermentable carbohydrates; they are centrally located, rarely subterminal, and do not swell the vegatative cell in which they are formed. Isolated colonies in deep agar are compact, opaque, white or grayish white biconvex discs. On blood agar the round, smooth, opaque, entire-edged colonies are relatively large, 2 to 5 mm. in diameter, and surrounded by a zone of hemolysis.

Physiology. *Cl. welchii* is a strict anaerobe and grows readily in deep brain, meat infusion broth, agar, and gelatin mediums. Growth in sugar-free mediums is restricted. Optimum conditions are provided by mediums containing fermentable carbohydrates, but such cultures are often short-lived because of the lack of spore formation and the destructive action of the formed acids on the vegetative cells. Brain and meat mediums are not blackened normally, but the presence of metallic iron produces a distinct discoloration. Gelatin is liquefied, but coagulated serum or egg is not digested. Hydrogen sulfide is produced, indol is usually said to be negative, but its formation is uncertain.

Nutritive requirements are complex, and semisynthetic mediums which support growth include casein hydrolysate or nineteen amino acids together with pantothenic acid, thiamin, nicotinic acid, riboflavin, biotin, folic acid, pyridoxine, adenine, guanine, uracil, inorganic salts including those of manganese and iron, together with glucose. The production of α-toxin (see below) requires two additional substances, one found in enzymatic digests of certain proteins, and the other an alcohol-soluble constituent of pancreas; glyceryl phosphocholine appears to be in part responsible for the activity of the latter.

Acid and gas are produced in glucose, maltose, lactose, and sucrose; neither mannitol nor salicin is fermented; some strains ferment inulin and some glycerol. Broth cultures containing fermentable sugars become markedly turbid with abundant gas formation, and many cultures, possibly all at certain stages, become stringy and viscid. Milk is fermented with a characteristic "stormy" evolution of gas, followed by coagulation of the casein due to acid formation, and the curd is shortly torn to shreds by the continued evolution of gas within. The curd is not digested. This "typical" reaction is considerably modified by incomplete anaerobiosis; gas production may be slow, and the solid curd is torn only slightly if at all. Under optimum conditions 3.8 times the volume of the milk may be evolved as gas; hydrogen predominates during the early stages of the fermentation and carbon dioxide in the later stages.

Types. Although *Cl. welchii* strains from gaseous gangrene form the same toxin, they are not immunologically homogenous. Henderson[37] has observed that the Wilsdon types (see below) are homogenous with respect to heat-stable antigen with the exception of type D, in which he found six kinds of antigens in thirteen strains. Rodwell[73] has shown that a number of subtypes of each of the Wilsdon types may be distinguished by agglutination and precipitin tests, and there are some cross-reactions between types. The common antigen appears to be a capsular polysaccharide; this substance has been prepared by Svec and McCoy.[82] As yet, however, serological identification of *Cl. welchii* is not practical, and gel-diffusion studies of the soluble antigens of the several types has indicated that the group tends to be heterogeneous, and there is considerable strain variation within type.[26] Four biochemical types have been suggested on the basis of differences in the fermentation of glycerol and inulin, but these fermentative differences do not appear to be correlated with other variable characteristics.

Toxigenic bacilli closely resembling *Cl. welchii* and having immunologically related toxins have been isolated from lower animals. These are the lamb dysen-

tery bacillus (*B. agni*), a bacillus causing a disease of sheep called "struck" (*B. paludis*), and a bacillus responsible for an enterotoxemia of sheep (*B. ovitoxicus*). Wilsdon proposed that these be designated *Cl. welchii* but that four types be distinguished: *Cl. welchii* type A—the gaseous gangrene bacillus; *Cl. welchii* type B—the lamb dysentery bacillus; *Cl. welchii* type C—the "struck" bacillus; and *Cl. welchii* type D— the bacillus of enterotoxemia of sheep. Two other differentiable types were described subsequently. That designated type E has been found to be a cause of enterotoxemia of lambs and calves, and type F produces enterotoxemia of man, enteritis necroticans. Unless otherwise indicated, however, the name *Cl. welchii* refers to the gas gangrene bacillus, or type A.

Toxins. It has been shown that, as a group, these bacilli form a number of immunologically distinct toxins and that the observed inter-relationships of the toxins formed by the various types are attributable to the sharing of one or more of these components.[61] The effects produced by these toxins are as follows:

The α-toxin is hemolytic, lethal to mice on intravenous injection, and produces necrosis on intradermal injection into guinea pigs and rabbits. Termed by Wilsdon the W factor and designated by some workers as the ζ-toxin.

The β-toxin is not hemolytic, mice injected intravenously develop spasmodic twitchings and die almost immediately, and it produces skin necrosis in guinea pigs and rabbits. Termed by Wilsdon the Z factor.

The γ-toxin is not hemolytic, does not produce skin necrosis in guinea pigs, and is lethal to mice.

The δ-toxin is hemolytic but is not lethal to mice and does not produce skin necrosis.

The ϵ-toxin is not hemolytic, but produces skin necrosis in guinea pigs and rabbits and is lethal to mice. Termed by Wilsdon the X factor.

The θ-toxin is hemolytic and lethal and probably produces necrosis in high concentrations. It is oxygen-labile and thermolabile and very similar in properties and immunological specificity, though not identical with streptolysin O. It is the same as Prigge's toxin.

The η-toxin has lethal activity only. It has been found in only one strain (Lechien) of type A as yet examined, and its occurrence or absence in the other types is not definitely established.

The κ-toxin is a collagenase and is necrotic on intradermal inoculation and lethal on intravenous inoculation in the rabbit. A related but differentiable activity, the λ-toxin, is not active on collagen but on altered collagen such as hide powder.

The ι-toxin is a lethal toxin, apparently formed as a prototoxin and activated by protease activity; it is only transiently present in the growing culture.

The λ-substance is not a toxin but a proteolytic enzyme whose presence or absence is associated with type.

The μ-toxin is the name given to hyaluronidase formed by these bacilli.

The ν-substance is a deoxyribonuclease which is formed by all of the types of *Cl. welchii*.

The optimal conditions of pH, incubation time, and composition of the medium differ from one toxin to another, and a type producing more than one toxin will produce only those for which the conditions are optimal. The distribution among the Wilsdon types of the ability to form these toxins is indicated in the accompanying table. The terminology of the toxins has been somewhat confused owing to differences in the terms used by various workers; that used here has been generally agreed upon.

Of these the α-toxin has been of greatest interest since it is associated with the virulence of the bacilli. It is a hot-cold lysin, and hemolysis is demonstrable by incubation at 37° C. for 30 minutes, followed by chilling at 2 to 4° C. The hemolysis is dependent upon the presence of calcium or magnesium (*viz.*, 0.0025 M calcium acetate) and is, therefore, inhibited by phosphate. The α-toxin is also a lecithinase. It was observed independently by Nagler and by Seiffert in 1939 that the addition of toxic filtrate to human serum produces an opalescence, and this is known as the Nagler reaction. The opalescence is due to the splitting of lipoprotein with the liberation of free fat and has been developed as a quantitative assay of the activity by turbidity produced in saline extract of egg yolk, "lecithovitellin."[38] The free lecithin is hydrolized to phosphocholine and stearyloleylglyceride; since the phosphocholine is water-soluble, the activity may be estimated by measurement of the liberated water-soluble phosphorus under standard conditions.[50] The lecithinase activity occurs at 37° C. and, unlike hemolysis, does not require subse-

quent chilling. The hypothesis that hemolysis is due to alteration of the erythrocyte membrane by the hydrolysis of contained lecithin is an attractive one but does not account for the necessity for subsequent chilling for hemolysis to occur. However this may be, the α-toxin has been purified by methanol precipitation in the cold and effectively separated from the other toxins of *Cl. welchii*; lecithinase activity, lethal activity, and hemolytic activity remained proportional, suggesting that all are activities of the same substance, but the preparations were electrophoretically heterogeneous.

The θ-toxin[64] is sharply differentiable from the α-toxin in that it is an oxygen-labile hemolysin, *i.e.*, is inactive in the reduced state, is more rapidly absorbed on sheep erythrocytes in the cold than the α-toxin, and does not require calcium or magnesium for hemolysis and is, therefore, active in the presence of phosphate.

In addition to the α-toxin and θ-toxin, the κ-toxin breaks down muscle by dissolution of its collagen and reticulin structure; the part it plays in the pathology of the infection is not clear. It does not appear to be as important as the α-toxin, for while antiserum to the α-toxin alone is protective, anticollagenase alone is not.[3]

In general the toxins formed by *Cl. welchii* appear to account in very large part for the observed histopathology. Robb-Smith,[71] for example, has compared that in naturally occurring infection in man, in experimentally infected animals, and in normal human muscle exposed to the action of filtrates *in vitro* and found the histopathological changes substantially the same in all three.

There appears to be no simple method of typing *Cl. welchii*, and the presence or absence of the various toxins must be demonstrated. The typing procedure, using monospecific antiserums, is that of Oakley and Warrack referred to above, but strains do not always conform precisely to the general pattern,[9] and cross neutralization of the toxins may be incomplete.[13]

Pathogenicity for Man. *Cl. welchii* is, perhaps, the most important cause of gaseous gangrene and is found either alone or mixed with other anaerobes in the majority of cases of this disease. Tissue injury is a usual, perhaps essential, preliminary to infection, but once the bacilli are established they invade the surrounding tissue rapidly. They apparently travel along the interstitial tissue of the muscle and are often found beyond the gangrenous area. The large amount of hyaluronidase produced would seem to be related to this rapid spread, but a number of studies have indicated that there is little or no relation between the invasiveness of *Cl. welchii* and at least *in vitro* titers of hyaluronidase.

Although most commonly found in gangrene, *Cl. welchii* has also been observed in closed abscesses in uterine infections and in infections of the gastrointestinal, genitourinary, and biliary tracts. It has been isolated from the blood during life, but septicemia is much less common in man than in experimental animals, although blood invasion occurs frequently in man during the agonal period or immediately following death.

Toxins Formed by the Clostridium Welchii Types

TYPES	TOXINS											
	α	β	γ	δ	ϵ	θ	η	ι	κ	λ	μ	ν
Type A (gaseous gangrene bacillus)	+	−	−	−	−	±	±	−	+	−	±	+
Type B (*Bacillus agni*)	+	+	+	+	+	+	?	−	−	+	+	+
Type C (*Bacillus paludis*)	+	+	+	+	−	+	?	−	+	−	−	+
Type D (*Bacillus ovitoxicus*)	+	−	−	−	+	+	?	−	±	±	±	+
Type E (enterotoxemia)	+	−	−	−	−	+	?	+	+	+	−	+
Type F (enteritis necroticans)	+	+	+	−	−	−	?	−	−	−	−	+

± = variable from strain to strain.

Study of the "foamy organs" sometimes observed at autopsy has shown that the presence of gas in the internal organs shortly after death is often attributable to an invasion by this microorganism.

Evidence has accumulated that *Cl. welchii* may occasionally cause food poisoning, similar to food poisoning of other etiology, with an incubation period of 10 to 12 hours, vomiting and diarrhea, and recovery in 24 to 48 hours.[41, 84]

Cl. welchii is a normal inhabitant of the human intestine and is constantly present in small numbers; in fact, it has been used to a certain extent in Europe, as an indicator of fecal pollution of water. The toxemia of acute intestinal obstruction has been attributed by some to the proliferation of *Cl. welchii* in the bowel followed by absorption of formed toxin, but it is now clear that such a relationship does not exist. This bacterium is, however, found with some frequency in gangrenous appendicitis, and it has been reported that antitoxin is of value in the treatment of perforative appendicitis.

Since infection with *Cl. welchii* is frequently characterized by gross blood destruction, jaundice, and anemia, a possible relationship between this bacterium and various anemias has been of some interest. A pernicious and fatal anemia may be produced in experimental animals by intratibial inoculation of culture or a temporary but severe anemia by inoculation of filtrate. Both natural and experimental infections, therefore, lead to the development of severe anemia which is probably due to the continuous release of the hemolytic toxin.

Pathogenicity for Animals. Natural infections in lower animals with *Cl. welchii* types have been referred to above. The occurrence of the gas-gangrene bacillus, however, is rare; local abscesses have been observed in dogs and rabbits following injury. Experimentally certain strains are pathogenic for guinea pigs, pigeons, and mice, less so for rabbits. If a rabbit or guinea pig is killed a few minutes after intravenous injection of *Cl. welchii* and the body incubated at 37° C., gas is produced in a few hours through the body and the phenomenon of "foamy liver" reproduced. This phenomenon is not strictly specific for *Cl. welchii;* it may be produced by similar inoculations with several other anaerobes, though the results are less striking. The pigeon is susceptible and is used for the standardization of toxin and antitoxin.

The classic *welchii* toxin, the α-toxin, is not a powerful one; the MLD for a mouse is usually about 0.25 ml. of liquid culture. Antitoxins may be produced, which have both prophylactic and therapeutic value, and *welchii* is included in polyvalent antitoxic serums for gas gan-

Figure 165. *Clostridium novyi* from pure culture. The slight tendency to curvature is apparent in some of the vegetative cells. Note the subterminal spores and the absence of free spores in large numbers. Fuchsin; × 1050.

grene. Antitoxin to the α-toxin appears to be far more important than that to the θ-toxin. It is difficult to develop agglutinins for *Cl. welchii,* and an antibacterial immunity does not protect against infection.

CLOSTRIDIUM NOVYI (CLOSTRIDIUM OEDEMATIENS)

The third important anaerobe in gaseous gangrene was probably first discovered by Novy in 1894 in a study of "malignant edema" in guinea pigs, and was designated *Bacillus oedematis maligni Nr. II.* It was named *B. novyi* by Migula in 1900. In 1915 Weinberg and Séguin isolated several strains of this bacillus, but first regarded it as a new species and named it *B. oedematiens.* The French name has been used by European workers.

Cl. novyi is noteworthy not only for its importance in gaseous gangrene but also because of its strong, soluble exotoxin, which compares in potency with the toxin of the diphtheria and tetanus bacilli, and for which a correspondingly potent antitoxin can be produced.

Morphology. Novy's bacillus is a large, relatively thick rod, 2.5 to 10 μ in length and 0.8 to 1 μ in breadth, and occurs singly and in chains. In cultures navicular and curved forms are found; in animal transudates the shorter form predominates. Its numerous spiral flagella, which often become tangled in "bouquets," have been emphasized in nearly all the published descriptions. The rod is nonmotile under ordinary conditions of examination, for movement is markedly inhibited in the presence of air. Subterminal spores are produced but sparsely as a rule and best in nonfermentable mediums. The bacillus is gram-positive.

Young colonies in deep dextrose agar have a yellowish, opaque, irregular center surrounded by a delicate corona of short filaments. Later the colony clears, the center becomes cloudy, and is surrounded in 48 hours with a corona of tangled filaments. Surface colonies are extremely delicate, flattened, transparent, bluish gray, with irregular contours, and there is slight hemolysis on blood agar.

Physiology. *Cl. novyi* is a strict anaerobe and grows well at 37° C. in ordinary mediums and especially abundantly in the presence of a fermentable sugar. Meat and brain are not darkened; the former may be turned slightly pink or bleached. Gelatin is liquefied, but coagulated serum and egg are not digested. Nitrates are not reduced and indol is not formed, but hydrogen sulfide is produced. Acid is produced very slowly in litmus milk; after 10 to 30 days' incubation a fine flocculent clot appears which is not digested. Dextrose is fermented but lactose is not; the latter property serves to differentiate *Cl. novyi* from *Cl. septicum* and *Cl. chauvoei.* There is not complete agreement as to the other fermentation reactions.

Antigenic Structure and Toxin. Three immunological types of *Cl. novyi* have been defined and designated type A, type B and type C. These correspond to types based on toxin formation (see below). *Cl. hemolyticum,* the etiologic agent of infectious icterohemoglobinuria of cattle, is considered to be *Cl. novyi* type D since it shares the β-toxin with the other types.[62]

The toxin formed by this organism is the most potent of the gaseous gangrene bacilli toxins; in contrast to *Cl. welchii* whose filtrates contain 4 to 5 mouse MLD per ml. the lethal dose of *Cl. novyi* filtrate for the mouse is about 0.005 ml. Lecithinase and hemolysin activity are present, and six components have been identified by Oakley, Warrack, and Clarke.[63] These are designated the α-, β-, γ-, δ-, ϵ-, and ζ-toxins of which the α-toxin is the classic lethal toxin. The α-toxin is associated with a lipase activity which is responsible for the pearly layered colonial morphology. The lecithinase activity is biochemically similar to that of the α-toxin of *Cl. welchii,* but the lecithinases of types A and B are distinct serologically from one another and from the α-toxin of *Cl. welchii.* The dis-

tribution of the toxins corresponds to the immunological types noted above and is illustrated in the accompanying table from Oakley et al.

Distribution of Toxins Among Clostridium Novyi Types

ACTIVITY OF TOXIN	DESIGNATION	CL. NOVYI TYPES		
		Type A	Type B	Type C
Lethal, necrotizing	α	+	+	−
Hemolytic, necrotizing, lecithinase	β	−	+	−
Hemolytic lecithinase	γ	+	−	+?
Oxygen-labilehemolysin	δ	+	−	−
Opalescence in lecithovitellin	ϵ	+	−	−
Hemolysin	ζ	−?	+	−

Pathogenicity for Man. The relatively frequent occurrence of *Cl. novyi* in gaseous gangrene has been noted earlier. The disease is characteristically a toxemia, although septicemia is not rare. Like *Cl. welchii,* Novy's bacillus is often a terminal invader. In pure infections there is less tissue destruction than with *Cl. welchii* or *Cl. septicum.* The postmortem findings consist mainly in a massive localized edema, with neither the extensive gas production of the former nor the sanguineous necrosis of the latter.

Pathogenicity for Animals. Natural infections due to *Cl. novyi* have been observed in guinea pigs, cattle, horses, and hogs. There is some evidence suggesting that Type B is etiologically related to infectious necrotic hepatitis (black disease) of sheep. Guinea pigs, rabbits, rats, mice, cats, sheep, horses, and pigeons are susceptible to small doses of culture. Subcutaneous, intramuscular, and intravenous inoculations reproduce the disease experimentally. Toxicity and pathogenicity are easily lost; Novy's original strain, which still survives in several laboratories, has long since failed to kill experimental animals. This is true also of strains isolated within 5 to 10 years.

The action of the toxin freed from bacteria is very similar to that of whole cultures. Sublethal, subcutaneous doses of toxin or culture produce a peculiar nonhemorrhagic, gelatinous local edema which reaches its maximum in 2 or 3 days. It may be followed by small, superficial hemorrhages, after which it is slowly absorbed, leaving a slightly sclerotic scar. Such lesions appear not to form open phlegmons, as in the case of *Cl. welchii* cultures, and may also be contrasted with those of the vibrion septique and *Cl. chauvoei,* which, if they appear at all, are always fatal. Washed cultures are harmless.

Antitoxin has been produced in rabbits, sheep, and horses by successively increased doses of toxic filtrates. The antitoxin has prophylactic and, to some extent, therapeutic value under experimental conditions, and is now represented in several polyvalent American serums for anaerobic infections.

CLOSTRIDIUM HISTOLYTICUM

Among the species of bacteria discovered by Weinberg and Séguin in war wounds, none is of more interest than *Cl. histolyticum,* so named because of its remarkable liquefying action upon living tissues. It may be somewhat more common in gaseous gangrene than indicated earlier. It has also been recovered from soil and human feces and from poisoned arrows.

Morphology. *Cl. histolyticum* is a gram-positive motile rod, 3 to 5 μ long and 0.5 to 0.7 μ wide, that forms subterminal clostridial spores. In smears from lesions it appears generally in the form of single or paired short rods with rounded ends. The flagella, often more than twenty in number, are peritrichal. Deep agar colonies vary, according to the consistency of the medium, from compact lobulate globules in 2 per cent agar to fluffy semitransparent or even cottony balls in lower concentrations. Surface colonies are minute, round dewdrops and are hemolytic on blood agar.

Figure 166. *Clostridium histolyticum* from pure culture. Note the characteristic short rods with rounded ends and the clostridial subterminal spores. Fuchsin; × 1050.

Physiology. Originally described as an obligate anaerobe, *Cl. histolyticum* is capable of a delicate transparent growth upon the surface of meat infusion agar and is perhaps best regarded as microaerophilic or as a facultative anaerobe.

This bacillus is actively proteolytic; not only is gelatin liquefied, but meat and brain and coagulated serum and egg are digested. In older cultures a precipitate of tyrosine crystals appears. Nitrates are not reduced and indol is not formed. No carbohydrates are known to be fermented in spite of statements to the contrary regarding dextrose. The action on milk is slow, but after several days a soft clot is usually formed and then slowly digested, and *Cl. histolyticum* is a proteolytic type.

Toxins. A number of toxic substances are formed, a lethal and necrotizing α-toxin, and several proteolytic enzymes. One of these, the β-toxin, is a collagenase, and another, the γ-toxin is a cysteine-activated enzyme which attacks altered collagen, *viz.*, azocoll.[60] A third, designated the δ-toxin, is a serologically distinct protease.[51]

Pathogenicity. Infection with *Cl. histolyticum* alone is probably a rare occurrence; mixed cultures with other anaerobes and aerobes appear to be the rule both in war wounds and in infections observed in horses. Most pure cultures of this bacillus are pathogenic under experimental conditions for rabbits, guinea pigs, mice, and rats, but there is considerable difference between strains. Subcutaneous inoculation of 1 or 2 ml. of a 24-hour broth culture generally produces a local tumefaction followed in 24 to 48 hours by complete sloughing of the overlying skin; then, as a rule, slow healing occurs. Intramuscular inoculation causes swelling, followed by progressive myolysis. If the gluteus muscle of a guinea pig is selected for the inoculation, it may be entirely denuded from the bone within 24 to 48 hours. The tissues literally drip away, and in some cases the limb may be disarticulated. Curiously there is often little or no intoxication of the animal, but death usually follows through peritonitis due to perforation of the peritoneum. There is occasionally invasion of the blood stream, but generally septicemia does not occur. There is no gas formation in such pure infections.

Bacteria-free filtrates have a lytic action which can be demonstrated if sufficiently large quantities (5 ml.) are injected. The most characteristic effect is the formation of a sterile hematoma filled with uncoagulated blood in which the red corpuscles are still intact. Deep intramuscular inoculation produces an edema which disorganizes and separates the tissue, while gross lesions are observed only occasionally following intravenous inoculation. Three toxins have been distinguished: an α-toxin that is lethal and necrotizing, a collagenase or

β-toxin, and a γ-toxin that acts upon altered collagen but not upon collagen. Agglutinating and antitoxic serums have been produced.

CLOSTRIDIUM SPOROGENES

Cl. sporogenes, which in pure culture is a harmless saprophyte, is included here because it is frequently associated with the pathogenic anaerobes in mixed gangrenous infections, very possibly because it is so widely distributed in nature. It has frequently been confused with the pathogenic forms; not only have cultures labeled something else proved to be *sporogenes,* but there has been a tendency to regard "atoxic variants" or "atoxic strains" of pathogens as *Cl. sporogenes.* On the other hand, the presence of "atoxic variants" of pathogenic species is in many cases attributable to mixed cultures containing *sporogenes.* The spores of this microorganism are unusually resistant and invariably survive with those of the pathogens or even after the pathogenic spore-formers are killed in preliminary selective heating.

Morphology. *Cl. sporogenes* is an actively motile gram-positive, slender rod, 3 to 7 μ in length and 0.6 to 0.8 μ in breadth, with rounded ends. The cells occur individually, in pairs, in short chains, and sometimes in filaments. The spores are oval, eccentric to subterminal, and swell the vegetative cell. The flagella are peritrichal. The deep agar colonies have the appearance of woolly balls with a dense, compact center. Surface colonies on blood agar are hemolytic, transparent, and usually rhizoid, or ameboid, with a slightly raised center; they appear moist and at first may resemble minute dew drops.

Cl. sporogenes requires strictly anaerobic conditions for growth and will grow upon all the ordinary mediums. It has been cultivated in synthetic solutions containing tryptophan, leucine, tyrosine, arginine, and phenylalanine, together with an unknown substance termed "sporogenes vitamin." Its optimum temperature is 37° C., but it will grow at temperatures as high as 50° C.

This bacillus is actively proteolytic; it produces blackening and digestion of brain and meat mediums, coagulated egg, and serum. An excess of fermentable sugar delays or inhibits this proteolysis, and the presence of metallic iron or certain iron salts accelerates it. Tyrosine crystals are not obvious. Gelatin is liquefied and blackened, hydrogen sulfide is produced, but indol production is doubtful, and nitrates are not reduced to nitrites. Reports on sugar fermentations are conflicting. According to Bergey, acid and gas are formed from dextrose, levulose, galactose, and maltose, while lactose, sucrose, salicin, and inulin are not

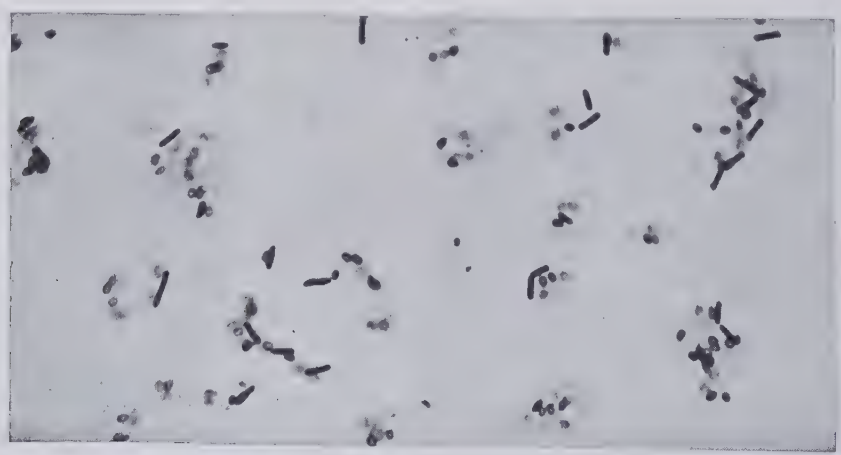


Figure 167. *Clostridium sporogenes* from pure culture. Note the close morphological resemblance of this species to the pathogenic forms. Fuchsin; × 1050.

fermented. Growth on milk is at first slow; in 48 to 72 hours a clot is formed and progressive liquefaction occurs with abundant gas and acid formation until the casein is completely peptonized.

Pathogenicity. There is no authentic record of a natural infection attributable to *Cl. sporogenes* alone. It has been claimed that this bacillus is a factor in certain intestinal disorders, but its frequent occurrence in the intestinal tract of healthy men and animals is not consistent with this.

In animal experiments relatively large doses are required to produce lesions; less than 5 ml. of a young dextrose broth culture injected subcutaneously in guinea pigs, which are the most susceptible experimental animals, usually results in only a local reaction. In a few hours the immediately overlying hair loosens, the skin becomes gangrenous and raised slightly over an area of subcutaneous tissue digestion in which a small amount of gas appears. Such animals usually show no systemic involvement, and the lesion heals in a few days, leaving a necrotic scar which heals slowly. The reaction to intramuscular injection is only slightly more severe.

The most obviously important of the pathogenic effects of *Cl. sporogenes* (and probably of other putrefactive anaerobes) is that of a mutual accleration in metabolism which occurs during growth with the pathogenic anaerobes, especially *Cl. welchii, Cl. septicum,* and *Cl. novyi.* While the presence of various aerobes is in some degree stimulating to the growth of obligate anaerobes (due partly, as Pasteur suggested, to the absorption of oxygen, but also to other factors), the presence of putrefactive anaerobes greatly enhances the pathogenicity of the nonputrefactive pathogens. The proteolytic forms supply protein-split products which the fermentative types are unable to elaborate so rapidly.

CLOSTRIDIUM CHAUVOEI (CLOSTRIDIUM FESERI)

Blackleg, also known as quarter evil and symptomatic anthrax (not to be confused with anthrax), is an acute disease affecting cattle. It occurs wherever cattle are kept and is prevalent throughout the United States with the possible exception of the Southern Atlantic and Eastern Gulf States. The name blackleg, like gaseous gangrene, has been applied to affections due to various anaerobes; in some instances *Cl. septicum* or, rarely, *Cl. novyi* is found, but the principal cause is *Cl. chauvoei* (*Cl. feseri*). Just as *Cl. welchii* (type A) is not involved in natural infections of lower animals, so *Cl. chauvoei* has never been shown to be responsible for any human infection.

Although the bacilli had been earlier observed and the disease transmitted by the injection of the serous fluid from an infected animal into a healthy animal, *Cl. chauvoei* was cultivated, and its causal relation to blackleg established by Arloing, Cornevin, and Thomas in 1887.

Morphology. *Cl. chauvoei* is a gram-positive, motile, sporulating rod. The size is variable, ranging from 3 to 8 μ in length and about 1 μ in breadth. The cells occur singly as a rule; in contrast with *Cl. septicum,* there is little tendency to form chains or filaments. The spores are subterminal and oval, swelling the vegetative cell in which they occur. Sporulation is often preceded by a marked swelling of the vegetative cell. Deep agar colonies are minute, compact, and downy. On the surface of blood agar well-separated colonies are flat, round or leaflike, and hemolytic.

Physiology. This bacillus is a strict anaerobe and, like *Cl. sporogenes,* grows at temperatures as high as 50° C., though the optimum is 37° C. It will grow on the usual laboratory mediums but is best cultivated in meat or brain medium. These are neither discolored nor digested by pure cultures, but they may be slightly softened. Gelatin is liquefied, but coagu-

Figure 168. *Clostridium chauvoei* from pure culture. Subterminal oval spores are apparent; note the swollen pre-sporulating cell on the far right. Fuchsin; × 1050.

lated serum and egg are not. Hydrogen sulfide is produced, but indol is not formed, and nitrates are not reduced to nitrites. Dextrose, levulose, galactose, maltose, sucrose, and lactose are fermented with the formation of acid and gas; inulin, salicin, mannitol, dulcitol, and glycerol are not fermented. It may be noted again that this microorganism may be differentiated from the closely related *Cl. septicum* on the basis of the sucrose and salicin fermentation. Litmus-milk cultures become acid and the casein is precipitated, but peptonization does not occur.

Pathogenicity. Natural infections due to *Cl. chauvoei* occur principally in cattle. There are still several obscure features in the epidemiology. The disease occurs at special seasons of the year, is connected with certain localities, and is said to show a distinct predilection for the best young stock. The portal of entry is uncertain; whether it enters by way of minute abrasions on the skin or through the gastrointestinal mucosa is quite unknown. Experimentally, *Cl. chauvoei* is pathogenic for cattle, sheep, goats, guinea pigs, and mice; horses, asses, hogs, rabbits, rats, and pigeons are somewhat refractory.

The symptoms in animals consist in crepitant localized swellings, which in natural infections occur on the thighs, neck, or shoulders. The animals become stupid, feverish, and anorectic. Treatment is rarely successful, and sick animals usually die in 1 to 2 days. The disease is a progressive bacteremia. A weak exotoxin is produced.

Immunization. An effective immunity is produced by inoculation with a formalin-inactivated whole culture vaccine or with washed bacteria inactivated with calcium chloride. An alum-precipitated toxoid has been used also. The immunity so produced is limited, and probably effective little longer than a year, but under natural conditions of exposure to infection may be reinforced to give the appearance of a long-lasting immunity. Unlike most of the other diseases of clostridial etiology, blackleg responds to treatment with antibiotics, and penicillin is most commonly used. A hyperimmune serum may be prepared by immunization of horses with vaccine which is said to have therapeutic value.

CLOSTRIDIUM BOTULINUM

Botulism was first definitely observed in Germany in 1785 and was and is associated, though by no means exclusively, with the consumption of sausages; hence the not altogether appropriate name botulism. The causative bacterium was isolated by van Ermengem in 1896 and named *Bacillus botulinus*. It is now known as *Cl. botulinum*.

Morphology. *Cl. botulinum* is a large, pleomorphic, gram-positive, motile, sporulating rod, 4 to 6 μ in length and 0.9 to 1.2 μ in breadth. The cells occur singly, in pairs, and in chains. There are four to eight peritrichal flagella. The spores are subterminal and oval and distend the vegetative cells containing them. Spore formation is variable from strain to strain, some strains producing spores abundantly, others sparsely; but in general spore formation is best in sugar-free mediums.

Deep agar colonies are translucent, globular, and diffuse, or flat and heart-shaped or disc-shaped, according to the consistency of the medium. Surface colonies are relatively large, 5 to 10 mm. in diameter, glistening, translucent at the edges with a thicker brownish center, filamentous, and hemolytic on blood agar.

Physiology. *Cl. botulinum* may be grown on the usual laboratory mediums under strict anaerobic conditions; cultivation on synthetic solutions has indicated that the amino acids cystine, leucine, lysine, glycine, and proline are required. Amino acids are decomposed by coupled oxidation-reduction reactions rather than by direct oxidation. Brain, meat, and coagulated protein mediums are blackened and digested; gelatin is liquefied. Milk is peptonized. Hydrogen sulfide is produced, but nitrates are not reduced to nitrites, and indol is not formed. Dextrose, levulose, and maltose are fermented; the fermentation of other sugars is variable from strain to strain and type to type. The spores are highly resistant and withstand boiling for 30 minutes to 22 hours, and autoclaving at 120° for as long as 20 minutes.

Irrespective of the presence of fermentable sugar, a potent soluble toxin is produced which resembles other soluble toxins in most respects. It is, however, unusually stable to heat; heating to 80° C. for 30 minutes or boiling for 10 minutes is required to destroy it. Botulinum toxin is unique in that it is not rapidly destroyed by the digestive enzymes of the gastrointestinal tract and hence is effective when given by mouth.[44, 45] It is the most potent bacterial toxin known; the guinea pig MLD may be as small as 1×10^{-6} ml. of broth culture.

Figure 169. *Clostridium botulinum* type A from pure culture. Note the subterminal swollen spores and free unstained spores admixed with the vegetative cells. Fuchsin; × 1050.

Types. *Cl. botulinum* is subdivided into a number of types which differ from one another in that their toxins are immunologically different. The best known of these are types A and B, which in the past have been regarded as solely responsible for human botulism. It is supposed that van Ermengem's original culture, now no longer available, was type B.

Additional types have been described since the early 1920's. A toxin-producing anaerobic bacillus isolated from fly larvae (the ingestion of which was associated with a paralytic disease of chickens) has been designated type C. A closely related bacillus was isolated from botulism of cattle in Australia which was designated *B. parabotulinus.* This bacillus is now designated *Cl. botulinum* type Cβ and the fly larvae bacillus *Cl. botulinum* type Cα. The toxins of these subtypes of type C are related in that Cα-antitoxin protects against both Cα- and Cβ-toxin, but Cβ-antitoxin protects against Cβ-toxin but not Cα-toxin.

A South African strain first described by Theiler and Robinson, designated by them *Cl. parabotulinum equi,* was further studied and given the name *Cl. botulinum* type D, for its toxin is not neutralized by the antitoxins of types A, B, or C. There is some confusion in this nomenclature in that the type designated type D by the French workers corresponds to type C in this country. Still another type, *Cl. botulinum* type E, has been isolated in Russia from fish, consumption of which resulted in human botulism. The type E toxin is not neutralized by antitoxins of the other four types. There appear to be some strain differences in the action of type E toxins though complete cross-neutralization occurs. Type E has been found as a cause of human botulism in the United States and Canada, in France and in Japan.

Toxins. As just indicated, a number of immunologically different toxins are produced by *Cl. botulinum,* and a given toxin is produced by a single type rather than the various toxins being distributed among the types as in the case of *Cl. welchii.* Furthermore, the pharmacological action of the toxins is essentially the same and differentiation can be made only on an immunological basis, *i.e.,* the passive protection test in which a series of experimental animals is passively immunized, each with a single antitoxin, and all challenged with the unknown toxin.

Of these toxins, that of type A is the most potent, exceeds in toxicity the other soluble toxins such as those of the diphtheria and tetanus bacilli, and is the most potent toxic substance known. The botulinum toxins, though protein in nature, are relatively resistant to proteolytic digestion and are thus effective when given by mouth. The type A toxin has been prepared in crystalline form by alcoholic precipitation in the cold, and by sodium sulfate and acid precipitation. The preparations are apparently pure protein with the properties of a globulin and a molecular weight of 9 to 11×10^5. The LD_{50} dose for the mouse contains 4.5×10^{-9} mg. of nitrogen and consists of about 2.1×10^7 molecules. Type B toxin has also been prepared as a pure homogeneous protein. It is somewhat less potent than type A toxin, and the mouse LD_{50} dose contains 5 to 9×10^{-9} mg. nitrogen. These toxins are readily prepared in highly purified form by preliminary precipitation at pH 3.5 with mineral acid, and subsequently by ethanol in the cold.[22, 23] Substantially this procedure has been applied to the preparation of purified toxins of type C,[14] D,[15] and E,[31] and the last has also been purified in ion exchange cellulose columns.[30] Both pure and crude botulinum toxins may be detoxified with formaldehyde to give formol toxoid which may be used for active immunization.[90]

The types of *Cl. botulinum* are not biochemically or culturally distinguishable, but, as a group, they may be divided into two biochemical types, the one proteolytic (sometimes designated ovolytic but digesting proteins other than coagulated egg albumin), whose cultural characteristics have been described above, and the other saccharolytic or fermentative in character, whose members do not hydrolyze coagulated native proteins. The proteolytic group includes type A and some

strains of type B (the majority of the American type B strains are proteolytic, while a great many of the European type B strains are not). The nonproteolytic group includes some strains of type B, and, so far as is known, all strains of types C, D, and E. It has been suggested that only the nonproteolytic varieties be designated *Cl. botulinum* and that the proteolytic varieties be termed *Cl. parabotulinum*.

Pathogenicity for Man.[53] Human botulism is almost invariably the result of eating preserved foods in which the bacillus has grown and produced toxin. In Europe most cases have been due to the consumption of various kinds of preserved meats, such as sausage, ham, potted goose, or duck, while in the United States the incriminated foods have been canned vegetables for the most part. There are surprisingly few cases of botulism in this country in view of the ubiquitous distribution of *Cl. botulinum* spores in the soil—type A has been found most commonly in the Rocky Mountain and Pacific Coast states, while type B predominates in the Mississippi Valley, Great Lakes region, and Atlantic Coast states. Type A predominates in English soils, though type B may be found also. According to Meyer, in the period 1899 to 1954, there have been 514 outbreaks in the United States, with 1350 cases and 861 deaths. The incidence is generally low; it rose to a peak of twenty-three outbreaks in 1922, declined and rose again to a peak of twenty-six in 1935, and has since remained relatively high but with some tendency to fall. Type E botulism has occurred a number of times in Canada since the type was described; 32 outbreaks involving 145 persons with 56 deaths were reported to 1957.[20, 21] Home-canned foods are now almost always responsible, and there has been no outbreak attributable to the consumption of commercially canned foods in this country since 1925, with the exception of a single outbreak of type E that resulted from the consumption of food imported, but prepared in this country.

As in the case of the other sporulating anaerobes, the disease produced by *Cl. botulinum* is an intoxication; in botulism, in fact, there is no invasion of the tissues and the toxin is preformed outside the body. Under experimental conditions in which massive doses of spores have been injected, it is probable that no infection has been set up. *Cl. botulinum* has, however, been found in contaminated wounds in mixed culture with aerobic and anaerobic bacteria. Under rare circumstances, then, it may proliferate in the tissues. In a group of three cases there were no symptoms of botulism, but in three fatal cases in which type A was found, symptoms of botulism were present.[19, 35, 36, 85]

Human botulism is most commonly caused by types A and B. The pharmacological activity of these toxins seems to be substantially identical, and the mechanism of their action has been of con-

Types of Clostridium Botulinum

TYPE	SYNONYM	BIOCHEMICAL CHARACTER	DISEASE	ANTITOXIN
A		Proteolytic	Botulism of man, limberneck of chickens	Specific
B		Some strains proteolytic	Botulism of man, limberneck of chickens	Specific
Cα	Fly larvae bacillus	Nonproteolytic	Paralytic disease of chickens, botulism of wild ducks	Neutralizes Cβ-toxin
Cβ	*Cl. parabotulinum*	Nonproteolytic	Forage poisoning of cattle (Australia)	Specific
D	*Cl. parabotulinum equi*	Nonproteolytic	Lamziekte of cattle (Africa)	Specific
E		Nonproteolytic	Botulism of man	Specific

siderable interest. Acetylcholine produces contraction of botulinum-poisoned muscle, but not of curare-poisoned muscle. In the poisoned animal apparently acetylcholine is not produced in the end plates, and the action of the toxin is proximal to the point at which it is produced. It has been found that the activity of the toxin is on the nerve filaments since acetylcholine is released following direct stimulation of the excised diaphragm of the guinea pig, but not following tetanization of the phrenic nerves. The evidence suggests that the neuromuscular paralysis observed in the poisoned animal is a result of interference with conduction in the terminal twigs of motor nerves, near or at the points of final branching, but proximal to the site of acetylcholine release.[10, 80] In man paralysis of the motor nerve end plates in the striated muscles and diaphragm is produced, and the symptoms include vomiting, constipation, ocular paresis, and pharyngeal paralysis. Death may occur within a day of the onset of symptoms or may be delayed for as long as a week. At autopsy the liver, kidneys, and meninges are congested, and there may be thrombosis. The case fatality is variable; in this country it has been 60 to 70 per cent but lower in Germany, perhaps 25 per cent, possibly owing to the greater prevalence of the somewhat less toxic type B.

Pathogenicity for Lower Animals. Associated with human cases of botulism there have been numerous outbreaks of limberneck, a paralytic disease, among fowls fed the toxin-containing food. Other forms of botulism in lower animals occur under natural conditions. *Cl. botulinum* types C and D appear to be associated exclusively with the disease in lower animals. Certain forms of forage poisoning in cattle and horses in Australia are botulism, but whether the bacilli grow and form toxin in the fodder or whether the disease results from the ingestion of rabbit carrion is not entirely clear. The South African disease of cattle, lamziekte, is botulism resulting from the ingestion of contaminated carrion. In the United States botulism of wild ducks and other waterfowl due to type Cα is prevalent and causes the death of thousands of ducks each year. The source of the toxin ingested by these fowl is uncertain.

Experimentally rabbits, guinea pigs, mice, monkeys, cats, and dogs are susceptible to toxin administered parenterally or *per os*. The symptoms are similar to those of naturally infected animals and of man, and the postmortem findings are much the same. Experimental animals vary widely in their susceptibility to the toxins of the various types of *Cl. botulinum*.

Immunity. Formol toxoid may be used as an immunizing antigen to produce an active immunity with circulating antitoxin present in the blood. Such active immunization has been carried out in lower animals when economically feasible; in Australia botulism of sheep and cattle has assumed sufficient proportions to justify such active immunization, and it has been applied on a small scale.

Man may also be immunized with fluid or alum-precipitated toxoid of type A or type B or a mixture of both types. With an immunization schedule of 0–2–10 weeks, the arbitrarily defined protection level of 0.02 units of type A and 0.005 units of type B antitoxin per ml. of circulating blood is reached in 50 per cent of those inoculated in about 3 months after initiation of the immunization. Booster inoculation at one year raises the titer 500-fold the minimal protective level, and the high titer persists for at least two years.[28] Under ordinary circumstances naturally occurring botulism in man is so rare that active immunization is not worth while.

Botulinum toxin is an excellent antigen and high-titer antitoxic serums may be produced. Under experimental conditions these antitoxins have marked prophylactic value, but their therapeutic efficacy is slight. It may be pointed out that in botulism, as in tetanus, the symptoms are a consequence of the injury to the nerve tissue, and the administration of antitoxin serves only to neutralize circulating toxin. The almost complete

lack of therapeutic effect of botulinum antitoxin in human botulism is undoubtedly attributable to the inevitable too-late administration.

REFERENCES

1. Abel, J. J., Warfield, M. F., and Chalian, W.: Researches on tetanus. IX. Further evidence to show that tetanus toxin is not carried to central neurons by way of the axis cylinders of motor nerves. Bull. Johns Hopkins Hosp., 1938, **63:**373–403.
2. Adriana, J., and Kerr, M.: Mephenesin and the combination of mephenesin and chlorpromazine in the management of tetanus: Report of 100 cases. Southern Med. J., 1955, **48:**858–862.
3. Aikat, B. K., and Dible, J. H.: The pathology of *Clostridium welchii* infection. J. Path. Bact., 1956, **71:**461–478.
4. Axnick, N. W., and Alexander, E. R.: Tetanus in the United States: A review of the problem. Amer. J. Pub. Health, 1957, **47:**1493–1501.
5. Baylis, J. H., et al.: The effect of transection on the ascent of tetanus toxin in the rabbit's spinal cord. J. Path. Bact., 1952, **64:**47–52.
6. Baylis, J. H., Mackintosh, J., Morgan, R. S., and Wright, G. P.: The effect of sclerosis of the nerve trunk on the ascent of tetanus toxin in the sciatic nerve of rabbits and on the development of local tetanus. J. Path. Bact., 1952, **64:** 33–45.
7. Berg, M., and Levinson, S. A.: Hyperglycemia induced in dogs by *Clostridium perfringens* toxin. Arch. Path , 1957, **64:**633–643.
8. Bernheimer, A. W.: Nutritional requirements and factors affecting production of toxin of *Clostridium septicum.* J. Exptl. Med., 1944, **80:**321–331.
9. Brooks, M. E., Sterne, M., and Warrack, G. H.: A re-assessment of the criteria used for type differentiation of *Clostridium perfringens.* J. Path. Bact., 1957, **74:**185–195.
10. Brooks, V. B.: Motor nerve filament block produced by botulinum toxin. Science, 1953, **117:** 334–335.
11. Brooks, V. B., Curtis, D. R., and Eccles, J. C.: Mode of action of tetanus toxin. Nature, 1955, **175:**120–121.
12. Brown, A., et al.: Value of a large dose of antitoxin in clinical tetanus. Lancet, 1960, **ii:** 227–230.
13. Bychenko, B. D., and Matveev, K. I.: Certain properties of *Clostridium perfringens* type A, B, C, D, E and F in relation to the typing of strains. J. Microbiol. Epidemiol. Immunobiol., 1959, **30:**69–76. (Translated from the Russian.)
14. Cardella, M. A., Duff, J. T., Gottfried, C., and Begel, J. S.: Studies on immunity to toxins of *Clostridium botulinum.* IV. Production and purification of type C toxin for conversion to toxoid. J. Bact., 1958, **75:**360–365.
15. Cardella, M. A., Wingfield, B. H., and Gottfried, C.: Studies on immunity to toxins of *Clostridium botulinum.* VI. Purification and detoxification of type D toxin and the immunological response to toxoid. J. Bact., 1960, **79:**372–378.
16. Clifton, C. E.: The utilization of amino acids and related compounds by *Clostridium tetani.* J. Bact., 1942, **44:**179–183.
17. Cohen, H., van Ramshorst, J. D., and Tasman, A.: Consistency in potency assay of tetanus toxoid in mice. Bull. W. H. O., 1959, **20:**1133–1150.
18. D'Antona, D.: La propagation de la toxine tétanique par la voie nerveuse démontrée par la résection des nerfs chez animaux en état d'immunité. Rev. d'Immunol., 1949, **13:**128–141.
19. Davis, J. B., Mattman, L. H., and Wiley, M.: *Clostridium botulinum* in a fatal wound infection. J. Amer. Med. Assn., 1951, **146:**646–648.
20. Dolman, C. E.: Recent observations on type E botulism. Canadian J. Pub. Health, 1957, **48:** 187–198.
21. Dolman, C. E.: Further outbreaks of botulism in Canada. Canadian Med. Assn. J., 1961, **84:** 191–200.
22. Duff, J. T., et al.: Studies on immunity to toxins of *Clostridium botulinum.* I. A simplified procedure for isolation of type A toxin. J. Bact., 1957, **73:**42–47.
23. Duff, J. T., et al.: Studies on immunity to toxins of *Clostridium botulinum.* II. Production and purification of type B toxin for toxoid. J. Bact., 1957, **73:**597–601.
24. Eckmann, L.: Therapeutische Massnahmen beim schweren Tetanus. Deut. med. Woch., 1955, **80:**684–687.
25. Elder, J. M., and Miles, A. A.: The action of the lethal toxins of gas-gangrene clostridia on capillary permeability. J. Path. Bact., 1957, **74:**133–145.
26. Ellner, P. D., and Bohan, C. D.: Serology of the soluble antigens of *Clostridium perfringens* types A-F by agar-gel diffusion. J. Bact., 1962, **83:**284–296.
27. Feeney, R. E., Mueller, J. H., and Miller, P. A.: Growth requirements of *Clostridium tetani.* III. A "synthetic" medium. J. Bact., 1943, **46:** 563–571.
28. Flock, M. A., et al.: Studies on immunity to toxins of *Clostridium botulinum.* VIII. Immunological response of man to purified bivalent AB botulinum toxoid. J. Immunol., 1962, **88:**277–283.
29. Fuller, R. M., and Ellerbeck, W.: Tetanus prophylaxis. J. Amer. Med. Assn., 1960, **174:**1–4.
30. Gerwing, J., Dolman, C. E., and Arnott, D. A.: Purification and activation of *Clostridium botulinum* type E toxin. J. Bact., 1961, **81:**819–822.
31. Gordon, M., Flock, M. A., Yarinsky, A., and Duff, J. T.: Studies on immunity to toxins of *Clostridium botulinum.* III. Preparation, purification, and detoxification of type E toxin. J. Bact., 1957, **74:**533–538.
32. Greenberg, L.: International standard for tetanus toxoid. Bull. W. H. O., 1953, **9:**837–842.
33. Greenberg, L.: The relative immunizing efficiency of tetanus toxoid preparations. Bull. W. H. O., 1955, **12:**761–768.
34. Gunnison, J. B.: Complement fixation reactions of the heat labile and heat stable antigens of *Clostridium tetani.* J. Immunol., 1947, **57:** 67–70.
35. Hall, I. C.: The occurrence of *Bacillus botulinus,* types A and B, in accidental wounds. J. Bact., 1945, **50:**213–217.
36. Hampson, C. R.: A case of probable botulism

due to wound infection. J. Bact., 1951, **61:**647.
37. Henderson, D. W.: The somatic antigens of the *Cl. welchii* group of organisms. J. Hyg., 1940, **40:**501–512.
38. van Heyningen, W. E.: The biochemistry of the gas gangrene toxins. I. Estimation of the toxin of *Cl. welchii,* type A. Biochem. J., 1941, **35:** 1246–1256.
39. van Heyningen, W. E.: The fixation of tetanus toxin by nervous tissue. J. Gen. Microbiol., 1959, **20:**291–300.
40. van Heyningen, W. E.: Tentative identification of the tetanus toxin receptor in nervous tissue. J. Gen. Microbiol., 1959, **20:**310–320.
41. Hobbs, B. C., et al.: *Clostridium welchii* food poisoning. J. Hyg., 1953, **51:**75–101.
42. Ikic, D.: Der Dynamik der Bildung von antitoxischen Einheiten nach erster Dosis des Anti-Tetanus Impstoffes. Ztschr. f. d. g. Hyg. u. ihre Grenzgebiete, 1961, **7:**230–236.
43. Kaufman, L., and Humphries, J. C.: Studies on the nutritional requirements of *Clostridium tetani.* I. A chemically defined medium. Appl. Microbiol., 1958, **6:**311–315.
44. Lamanna, C.: The most poisonous poison. What do we know about the toxin of botulism? What are the problems to be solved? Science, 1959, **130:**763–772.
45. Lamanna, C.: Oral poisoning by bacterial exotoxins exemplified in botulism. Ann. N. Y. Acad. Sci., 1960, **88:**1109–1114.
46. Lasson, H. C. A., Bjøreboe, M., Ibsen, B., and Neukirch, F.: Treatment of tetanus with curarisation, general anaesthesia, and intratracheal posture-pressure ventilation. Lancet, 1954, **ii:** 1040–1044.
47. Levine, L.: The quantitative flocculation reaction of equine tetanus antitoxin. Brit. J. Exptl. Path., 1959, **40:**343–357.
48. Looney, J. M., Edsall, G., Ipsen, J., Jr., and Chasen, W. H.: Persistence of antitoxin levels after tetanus-toxoid inoculation in adults, and effect of a booster dose after various intervals. New England J. Med., 1956, **254:**6–12.
49. Macfarlane, M. G.: On the biochemical mechanism of action of gas-gangrene toxins. Society for General Microbiology, Symposium No. 5, pp. 57–77. Cambridge University Press, 1955.
50. Macfarlane, M. G., and Knight, B. C. J. G.: The biochemistry of bacterial toxins. I. The lecithinase activity of *Cl. welchii* toxins. Biochem. J., 1941, **35:**884–902.
51. MacLennan, J. D.: The histotoxic Clostridial infections of man. Bact. Rev., 1962, **26:**177–274.
52. McClung, L. S.: The anaerobic bacteria with special reference to the genus Clostridium. Ann. Rev. Microbiol., 1956, **10:**173–192.
53. Meyer, K. F.: The status of botulism as a world health problem. Bull. W. H. O., 1956, **15:**281–298.
54. Miller, P. A., Eaton, M. D., and Gray, C. T.: Formation of tetanus toxin within the bacterial cell. J. Bact., 1959, **77:**733–740.
55. Moloney, P. J., and Hennessy, J. N.: Titration of tetanal toxins and toxoids by flocculation. J. Immunol., 1944, **48:**345–354.
56. Moussa, R. S.: Complexity of toxins from *Clostridium septicum* and *Clostridium chauvoei.* J. Bact., 1958, **76:**538–545.
57. Moussa, R. S.: Antigenic formulae for *Clostridium. septicum* and *Clostridium chauvoei.* J. Path. Bact., 1959, **77:**341–350.
58. Mueller, J. H., and Miller, P. A.: Unidentified nutrients in tetanus toxin production. J. Bact., 1948, **56:**219–233.
59. Oakley, C. L.: Gas gangrene. Brit. Med. Bull., 1954, **10:**52–58.
60. Oakley, C. L., and Warrack, H. G.: The alpha, beta, and gamma antigens of *Clostridium histolyticum.* J. Gen. Microbiol., 1950, **4:**365–373.
61. Oakley, C. L., and Warrack, G. H.: Routine typing of *Clostridium welchii.* J. Hyg., 1953, **51:**102–107.
62. Oakley, C. L., and Warrack, G. H.: The soluble antigens of *Clostridium oedematiens* type D (*Cl. haemolyticum*). J. Path. Bact., 1959, **78:** 543–551.
63. Oakley, C. L., Warrack, G. H., and Clarke, P. H.: The toxins of *Clostridium oedematiens* (*Cl. novyi*). J. Gen. Microbiol., 1947, **1:**91–107.
64. Orlans, E. S., Richards, C. B., and Jones, V. E.: *Clostridium welchii* epsilon-toxin and antitoxin. Immunol., 1960, **3:**28–44.
65. Perlstein, M. A., Stein, M. D., and Elam, H.: Routine treatment of tetanus. J. Amer. Med. Assn., 1960, **173:**1536–1541.
66. Pillemer, L., and Wartman, W. B.: The clinical behavior, incubation period, and pathology of tetanus induced in white Swiss mice by injection of crystalline tetanal toxin. J. Immunol., 1947, **55:**277–281.
67. Prausnitz, C.: Memoranda on the international standardization of therapeutic sera and bacterial products. League of Nations Health Organization, Geneva. 1929.
68. Ramon, G.: L'immunité conferée par l'anatoxine tétanique chez l'homme et chez le cheval. Précisions d'ordre immunologique et épidémiologique. Consequences. Rev. d'Immunol., 1939, **5:**477–490.
69. Regamey, R. H.: L'immunisation active contre le tétanos. Ergebn. Mikrobiol. Immunitätsf., 1959, **32:**270–381.
70. Reynaud, M.: Extraction de la toxine tétanique à partir des corps microbiens. Ann. Inst. Pasteur, 1951, **80:**356–377.
71. Robb-Smith, A. H. T.: Tissue changes induced by *Cl. welchii* type A filtrates. Lancet, 1945, **ii:**362–368.
72. Roberts, J. E.: Toxin production by *Clostridium perfringens.* I. Synthesis of α- and θ-hemolysins, lecithinase and proteinase in a synthetic medium. J. Bact., 1957, **74:**439–444.
73. Rodwell, A. W.: Serological subtypes in *Cl. welchii* types A, B, C and D. Australian Vet. J., 1941, **17:**58–68.
74. Schofield, F. D., Tucker, V. M., and Westbrook, G. R.: Neonatal tetanus in New Guinea. Effect of active immunization in pregnancy. Brit. Med. J., 1961, **ii:**785–789.
75. Shackleton, P.: The treatment of tetanus. Role of the anaesthetist. Lancet, 1954, **ii:**155–158.
76. Smith, L. DeS.: Clostridia in gas gangrene. Bact. Rev., 1949, **13:**233–254.
77. Smith, L. DeS.: Introduction to the Pathogenic Anaerobes. University of Chicago Press, 1955.
78. Stafford, E. S.: Active and passive antitetanus immunization. J. Amer. Med. Assn., 1960, **173:**539–541.
79. Stone, J. L.: On the mode of release of tetanus toxin from the bacterial cells. J. Bact., 1954, **67:**110–116.
80. Stover, J. H., Jr., Fingerman, M., and Forester, R. H.: Botulinum toxin and the motor end plate. Proc. Soc. Exptl. Biol. Med., 1953, **84:** 146–147.

81. Suri, J. C., and Rubbo, S. D.: Immunization against tetanus. J. Hyg., 1961, **59:**29–48.
82. Svec, M. H., and McCoy, E.: A chemical and immunological study of the capsular polysaccharide of *Clostridium perfringens*. J. Bact., 1944, **48:**31–44.
83. Symposium: Symposium on tetanus. Proc. Staff Meetings Mayo Clinic, 1957, **32:**141–167.
84. Taylor, C. N. D.: Food poisoning due to *Clostridium welchii*. Med. J. New Zealand, 1955, **54:**702–704.
85. Thomas, C. G., Jr., Keleber, M. F., and McKee, A. P.: Botulism, a complication of *Clostridium botulinum* wound infection. Arch. Path., 1951, **51:**623–628.
86. Veronesi, R.: Clinical observations on 712 cases of tetanus subject to four different methods of treatment: 18.2% mortality rate under a new method of treatment. Amer. J. Med. Sci., 1956, **232:**629–647.
87. Wright, E. A.: The effect of the injection of tetanus toxin into the central nervous system of rabbits. J. Immunol., 1953, **71:**41–44.
88. Wright, E. A.: The fixation of tetanus toxin to the brain stem of guinea-pigs. J. Path. Bact., 1954, **68:**131–135.
89. Wright, E. A., Morgan, R. S., and Wright, G. P.: The movements of toxin in the nervous system in experimental tetanus in rabbits. Brit. J. Exptl. Path., **32:**169–182.
90. Wright, G. G., et al.: Studies on immunity to toxins of *Clostridium botulinum*. V. Detoxification of purified type A and type B toxins, and the antigenicity of univalent and bivalent aluminum phosphate adsorbed toxins. J. Immunol., 1960, **84:**384–389.
91. Wright, G. P.: Botulinum and tetanus toxins. Society for General Microbiology, Symposium No. 5, pp. 78–102. Cambridge University Press, 1955.

Chapter 30

CORYNEBACTERIUM (THE DIPHTHERIA BACILLUS)

As a clinical entity diphtheria dates from the observations of Bretonneau in 1826. The diphtheria bacillus was observed and described by Klebs in 1883, but its etiological relation to the disease was suggested by the investigations of Löffler the following year. Löffler isolated the bacillus observed by Klebs in pure culture from a number of cases of diphtheria but expressly disclaimed the assumption that his bacillus was the causal agent of diphtheria, in part because he found it in the throat of a healthy child, and in part because he did not find it in all cases of what were apparently clinical diphtheria. The significance of Löffler's findings is now clear, however, for it is known that other bacteria, such as streptococci, can produce a condition in the throat closely resembling diphtheria and that the diphtheria bacillus is not infrequently present in the throat of healthy carriers. Further investigations by other workers indicated that the Klebs-Löffler bacillus was always present in the typical false membrane of diphtheria. In 1888 Roux and Yersin showed that this bacillus formed a soluble toxin which reproduced the characteristic symptoms and lesions of diphtheria and this demonstrated its etiological relation to the disease.

Morphology and Staining.[34] The diphtheria bacillus is a slender rod ranging from 1 to 6 μ in length and 0.3 to 0.8 μ in breadth. The bacilli are highly pleomorphic, for, in addition to the straight or slightly curved rods, club-shaped and branching forms are not infrequently observed. Septa in the cell and the occurrence of branched forms are readily apparent in the living cell examined by phase microscopy. The presence of the latter, which are a consequence of true branching, is indicative of the close relation of the diphtheria bacillus to some of the higher fungi. Upon completion of cell division a movement designated as snapping occurs, and the bacilli may remain attached but at sharp angles to one another.

The diphtheria bacillus exhibits a marked tendency to stain irregularly. Some cells stain solidly, others take the stain more deeply in transverse bands to give a barred appearance, and in still others deeply staining metachromatic or Babes-Ernst granules are found. A single cell may contain from one to generally not more than five or six such metachromatic granules; they may be found at one or both ends of the cells, particularly those with swollen ends, and when more than two are present the remainder are scattered within the cell substance. This irregular staining is apparent with Löffler's alkaline methylene blue or with toluidine blue.[14]

In stained smears the appearance of diphtheria bacilli is highly characteristic. They may not be identified on morphological grounds alone, however, for many

Figure 170. Colonies of *Corynebacterium diphtheriae* on blood agar. Note the smooth, raised, translucent appearance and relatively small size. × 2.

of the pseudodiphtheria bacilli or diphtheroids stain in the same irregular fashion and are similarly pleomorphic. It was formerly thought that there was an association between morphological type and virulence. At present little emphasis is placed upon morphology in this connection, though in a general way granular types seem to predominate in clinical diphtheria, and there appears to be a rough association between morphological type and the *mitis* and *gravis* types, as will appear. The diphtheria bacillus is gram-positive but decolorizes more readily than most of the gram-positive bacteria.

Surface colonies on Löffler's serum medium or on agar are small and gray; when viewed under low magnification they are found to be coarsely granular and somewhat irregular in outline, with ragged or fringed edges. On differential mediums containing potassium tellurite, colonies of the diphtheria bacillus are dark gray or black because of reduction of the tellurite and readily differentiated from those of contaminating bacteria. Tellurite reduction apparently occurs within the bacterial cell. Microscopic morphology may not be characteristic in smears made from tellurite medium cultures.

Physiology. The optimum growth temperature for the diphtheria bacillus is 34° to 36° C., and it grows well at 37° C.; growth will take place over the range from 15° to 40° C. An alkaline reaction is required, pH 7.8 to 8.0, and free access to air is essential, for growth under anaerobic conditions is sparse.* Growth and toxin production occur in aerated bulk, *e.g.*, "submerged," culture in tanks.[19]

In primary isolation the diphtheria bacillus is best cultivated on enriched mediums. Growth is rapid on Löffler's serum medium (3 parts of beef or sheep serum and 1 part of 1 per cent dextrose broth coagulated in slant form by inspissation), and minute but visible colonies appear after 12 to 24 hours' incubation. In recent years a variety of differential and selective mediums have been introduced, all of which contain potassium tellurite. The better known of these are the chocolate agar–tellurite medium of Anderson and his co-workers, which has been subject to minor modifications by a number of other workers, such as Neill's medium and Hoyle's medium which are used in England, and the various mediums developed by Clauberg, whose inspissated serum-glycerol-tellurite medium has been widely tested. There is general agreement that the proportion of positive cultures is somewhat higher with the Clauberg medium than with Löffler's medium; whether the heated blood-tellurite mediums are superior to Löffler's medium is not clear. As indi-

* Strains of virulent diphtheria bacilli which grow more luxuriantly under anaerobic conditions than in the presence of air have, however, been reported.

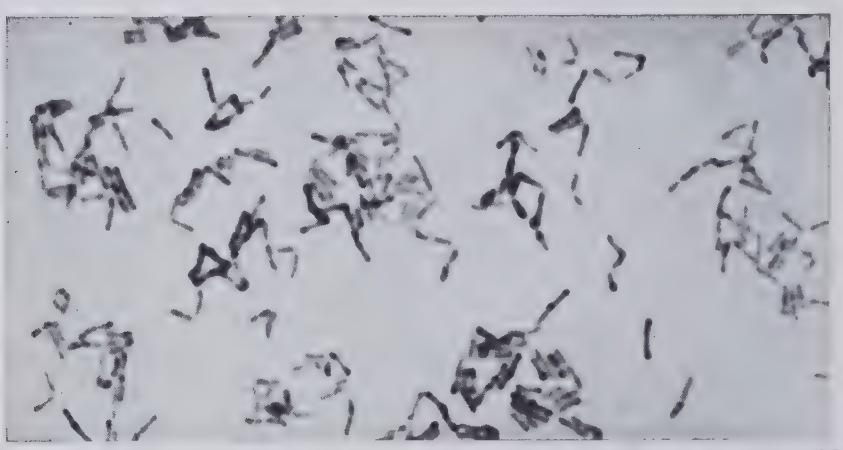


Figure 171. The diphtheria bacillus, *gravis* strain, pure culture on blood agar. Methylene blue stain. Note the bipolar staining and the club-shaped forms. The lightly stained cells with deeply stained areas are characteristic of *gravis* morphology. × 1200.

cated above, the characteristic morphology of the diphtheria bacillus is not always seen in smears from colonies on tellurite mediums, and, therefore, in some laboratories both Löffler's medium and a differential tellurite medium are inoculated and the former used for microscopic examination if typical colonies appear on the differential medium.

Enriched mediums are not, however, essential to the growth of the diphtheria bacillus, for this microorganism can be cultivated on ordinary nutrient and infusion mediums. Growth is somewhat scanty on the former but good on the fresh meat infusions. The intensive investigations of Mueller[51] have shed considerable light on the growth requirements of these bacilli. Some strains, including the well-known Park 8, may be cultivated on synthetic solutions containing a number of amino acids together with small quantities of nicotinic acid, β-alanine, or pantothenic acid, and pimelic acid. It has been suggested that pimelic acid is utilized by this bacterium for the synthesis of biotin since growth is stimulated by biotin in the absence of pimelic acid. Recently isolated strains also require oleic acid for development, especially if the inoculum is small. Nutritive requirements differ somewhat from one strain to another, and a general statement is not possible.

The diphtheria bacillus does not liquefy gelatin or digest coagulated protein. Indol is not formed,* nitrates are reduced to nitrites, and hydrogen sulfide is formed. All strains form acid but no gas from dextrose and levulose, and some strains ferment dextrin, glycogen, starch, galactose, maltose, and glycerol. There appear to be no well-defined biochemical groups among these bacilli. The fermentation of dextrose is of some interest in that propionic acid is formed. Other products of the fermentation include lactic, acetic, formic, and succinic acids and ethyl alcohol.[69]

In ordinary culture mediums the diphtheria bacillus may remain viable for relatively long periods. It will live 6 to 8 weeks on agar, 5 to 6 months on blood serum, 12 to 15 months on dextrose blood serum, and as long as 3 months in particles of diphtheritic membrane. Although virulence is ordinarily reduced by continued culture on laboratory mediums, some strains remain fully virulent, *i.e.*, toxigenic, on prolonged cultivation; Löffler recorded one instance in which virulence was maintained over seventy-seven transfers covering a period of 27 months. The bacilli are unusually susceptible to heat; a suspension or broth culture is killed by holding at 58° C. for 10 minutes. In diphtheritic membrane they are considerably more resistant.

* The test with sulfuric acid and potassium nitrite may be positive because of the formation of indol-acetic acid, but no color is produced with Ehrlich's reagent, *p*-dimethylamidobenzaldehyde.

Antigenic Structure. Although the diphtheria toxin (see below) is apparently immunologically identical in all strains, the diphtheria bacilli are heterogeneous with respect to cell antigens. They tend to agglutinate spontaneously, which may be partially countered by using 5 per cent saline as the suspending medium, but agglutinative types may be determined. They contain heat-stable somatic antigens which appear to be polysaccharide in nature, and heat-labile protein somatic antigens. The former have little differential value, and the agglutinative types which have been described depend upon the protein antigens.[39] There is some correlation between agglutinative types and the *mitis-intermedius-gravis* colonial type[33] (see below), but such agglutinative types have little significance to the disease, although antibacterial immunity is considered by some to be a possible element in effective immunity (see below).

Toxin. With the exception of the Shiga dysentery bacillus, the diphtheria bacillus is the only aerobic bacterium that produces a powerful exotoxin comparable to those formed by the sporulating anaerobes. Filtrates from broth cultures are not so toxic as those of the tetanus and botulinus bacilli; exceptionally potent filtrates may contain as much as 1000 guinea-pig MLD's per ml. Virulence is synonymous with toxigenicity, and the various virulence tests are tests for the formation of toxin.

The production of toxin by the diphtheria bacillus is markedly influenced by environmental and nutritive conditions; even strongly toxigenic strains may produce little or no toxin under unfavorable conditions. A slightly alkaline reaction, pH 7.8 to 8.0, is essential, for an acid reaction strongly inhibits toxin formation. Free access to air is also necessary, and for the production of toxin the bacilli are cultivated in thin layers of beef infusion broth. Maximum amounts of toxin are found after 7 to 10 days' incubation at 36° to 37° C. The toxicity is unstable to slight acidities, pH 6.0 or less, and is heat-labile and protein in nature.

An infusion medium, beef infusion, containing adequate amounts of peptone (2 per cent) has long been regarded as essential to the production of maximum amounts of toxin. The marketed brands of peptone are variable in this respect, some being much better for the production of toxin than others. The presence of protein or large peptone molecules is not essential to toxin formation, however, and potent toxins may be produced in chemically defined mediums containing appropriate amino acids and other compounds. The critical factor is not the quality of peptone or other source of nitrogen, as once thought, but the concentration of iron in the medium; the

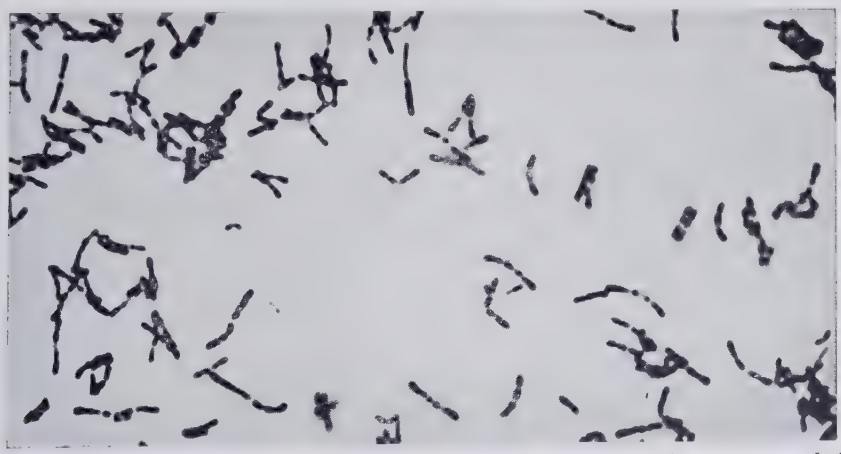


Figure 172. The diphtheria bacillus, *intermedius* strain, pure culture on blood agar. Methylene blue stain. Note the irregular staining and barred appearance characteristic of the *intermedius* variety. × 1200.

optimum being 0.14 μg. per ml., and 5.0 μg. per ml. almost completely inhibits its formation.

Toxin production lags behind growth and begins rapid accumulation in the medium when the bacterial population has reached near maximal levels, and concentrations of iron optimal for growth are in excess of that optimal for toxin production. Largely through the work of Pappenheimer,[55] a close relationship between cytochrome b and the succinoxidase system, and toxin formation has been established. Four mols of porphyrin and one mol of toxin disappear for every four mols of iron over the optimal concentration for toxin production. It is implied, on the one hand, that the toxin may be the protein moiety of an iron-porphyrin enzyme, and on the other, experimental evidence obtained from study of the metamorphosis of cecropia silkworm larvae has suggested that the toxin may interfere in the synthesis of such respiratory enzymes. The hypothesis is attractive and plausible, but as yet lacks supporting evidence for mammalian systems.

The toxin has been prepared as a crystalline protein and subjected by intensive investigation by Pope and Stevens.[58, 59, 60, 61, 62] This material is not immunologically homogeneous as indicated by the presence of agglutinin in homologous antibody whose removal by absorption did not affect antitoxic activity. Heterogeneity is also indicated by application of the gel-diffusion technique, and two antigens, in addition to that of the neurotoxin, are demonstrable and are also characterized by resistance to alkaline phosphate and pepsin.

Toxicity. The diphtheria toxin is a potent poison, though not as potent as the botulinum toxins, and is primarily a neurotoxin. Highly purified preparations have a molecular weight of about 72,000 and the lethal dose for the guinea pig is about 1 μg. Owing to unfortunate mistakes in the preparation of diphtheria prophylactics, it is known that about 12 guinea-pig MLD's are sufficient to kill a child. The dose-response curve in the guinea pig is very steep, so much so that the minimal lethal dose assay, adopted many years ago and persisting to the present, is practical. Assay of the toxicity is discussed below in connection with virulence tests and antitoxin neutralization. Like the other exotoxins, it is converted to toxoid by treatment with formaldehyde.

The pharmacological activity of the toxin is characteristic, and the essential features of the disease are reproducible experimentally with the toxin alone. It produces degenerative changes in the heart muscle, kidneys and liver, and peripheral nerves. The pathology in the adrenals is accentuated and characteristic in the guinea pig. The immediate cause of death in the acute disease is heart failure, and damage to peripheral nerves accounts for the post-diphtheritic paralysis observed in man, and in guinea pigs receiving near-fatal doses of toxin.

Diphtheria toxin is an excellent antigen and gives rise to high titer antitoxic serums. It has been noted from time to time that various substances, including bile, ascorbic acid (vitamin C), and sterols such as lanolin and cholesterol will neutralize diphtheria toxin. The significance of such observations is not entirely clear. Generally relatively large amounts are required to neutralize small quantities of toxin. One or two attempts have been made to utilize ascorbic acid in the treatment of clinical diphtheria but without significant results.

Lysogenicity and toxigenicity.[4] The observation that virulent, *i.e.*, toxigenic, diphtheria bacilli are lysogenic (Chap. 4), and that some nontoxigenic diphtheroid bacilli could often be made toxigenic by conversion to the lysogenic state, was made by Freeman[25] in 1951, and has been studied by a number of workers.[5, 13, 28, 29, 30, 31] It was found by Hewitt[35] that temperate phages producing lysogenic toxigenic diphtheria bacilli could be of heterologous origin, *i.e.*, staphylococci, and this significant observation has been extended by others.[15, 16, 17] Toxigenicity seems not to be a physiological character transmitted by phage-mediated transduction (Chap. 7); it is possibly an expression of an

altered metabolism, perhaps porphyrin metabolism, but as yet studies have been almost exclusively genetic rather than physiological. In any case, it is of interest that some 60 years after the etiology of diphtheria apparently had been fully established, it became evident that more than one microorganism is concerned.

The epidemiological implications are obvious, but are as yet unexplored. Thus the spread of the disease may be covert in some instances in the sense that it may depend in part on the dissemination of the appropriate phage, possibly carried in part by other bacteria such as staphylococci. In this connection it has been observed that there may be a geographical distribution of such phages.[32]

Variation. As in other groups of bacteria, smooth and rough variants of the diphtheria bacillus have been observed, and the type of colony formation has been found to be correlated with morphology and virulence; the S variant is the more virulent and the form commonly found in acute cases of diphtheria. Morphological and biochemical variation observed in the diphtheria bacilli has been critically reviewed by Morton.[50] As a consequence of its pleomorphism and tendency to branching and the like, the diphtheria bacillus has been thought by some to undergo the cyclic transformations of a complex life cycle including the production of gonidial and filterable forms. This concept is not generally accepted. It has also been suggested that some of the diphtheroid bacilli are diphtheria bacilli which have lost their ability to form toxin.

Types.[47] Morphological types of the diphtheria bacillus were described by Anderson and others in England in 1931 and since have been found in various parts of the world. These were of very considerable interest when first observed for there appeared to be an association, especially in England, between the type and the degree of severity in the clinical manifestations of the disease. Those designated as the *gravis* and *intermedius* types were found in severe cases of diphtheria, and the *mitis* type in the milder cases. The association was less clear on the Continent, and there appeared to be little or no relation in the United States, where the *mitis* type occurs much more frequently and perhaps only 1 per cent of the strains are of the *gravis* type. The toxins produced by the three types are equally neutralizable by the ordinary antitoxic serums, but the *mitis* strains produce toxin somewhat more actively *in vitro* than the *gravis* and *intermedius* types. The three types also appear to be equally virulent for the guinea pig. By now it is more or less generally agreed that the differentiation of these types is not significantly related to clinical se-

Figure 173. The varieties of the diphtheria bacillus on chocolate tellurite agar. *Left, mitis* type; note the characteristic raised, small black colony. *Center, intermedius* type; the lighter color, beginning radial striation and small size are apparent. *Right, gravis* type; the gray color, larger size, raised center, and radial striation are evident.

verity but has been useful from an epidemiological point of view.

These types may be differentiated by their colonial form on tellurite mediums. The *gravis* type produces irregular striated colonies predominantly gray in color; the *mitis* type, small, round, smooth, convex colonies predominantly black in color and softer in consistency; and the colonial form of the *intermedius* type lies between these. Colonial differences are also apparent on certain other mediums, such as trypsin-serum agar and a potato extract-cystine-water blue-glycerol medium devised by Clauberg. On fresh blood agar the *mitis* type is usually hemolytic, the *intermedius* type nonhemolytic, and the *gravis* type usually nonhemolytic. A further distinction is the fermentation of glycogen and starch by the *gravis* type, but other biochemical tests do not differentiate these types.

There is some association between colonial type and the morphology of the bacillary forms. Those of the *gravis* type show one or two deeply staining areas, the remainder of the cell staining very lightly; metachromatic granules are seldom observed. Bacilli of the *mitis* variety stain irregularly and contain very many well-developed metachromatic granules. The *intermedius* forms have the familiar barred appearance. Whereas some 80 per cent of the *intermedius* variety conform to this morphology, only 50 to 60 per cent of the *gravis* strains are typical, the remainder resembling the *mitis* and *intermedius* forms. Furthermore, 5 to 20 per cent of the *mitis* strains show barred forms.

Serological investigation has shown that while these three types are antigenically distinct from one another, the types are not necessarily homogeneous. The *mitis* strains are heterogeneous, the *gravis* strains fall for the most part into two types, and the *intermedius* strains are relatively homogeneous, some strains showing relationship to the *gravis* types.

Not all strains showing the morphological and biochemical characteristics of these types are virulent, *i.e.*, toxigenic, diphtheria bacilli. Since the diphtheria bacillus is differentiated on the basis of the formation of immunologically specific toxin, it is apparent that *mitis*, *gravis*, and *intermedius* types of diph-

Characteristics of the Diphtheria Bacillus Types

TYPE			*mitis*	*intermedius*	*gravis*
Morphology	Microscopic		Usually long, with many metachromatic granules—80 per cent typical	Usually barred, club forms common—80 pcer cent typical	Short, evenly staining—50–60 per cent typical
	Colonial	Tellurite	Small, round, smooth, convex, black with grayish periphery	Small, flat, dull, gray, raised center	Large, irregular, dull, gray, raised center, radial striations
		Chocolate	Smooth, semiopaque, glistening	Flat, dry, opaque, slight greenish zone	Flat, dry, matt, opaque
		Broth	Uniform turbidity, sometimes slightly granular, soft pellicle	Finely granular turbidity	Granular, flakes, pellicle-variable
Physiology	Fermentation of	Glycogen	—	—	+
		Starch	—	—	+
Immunology	Hemolysis		+	—	±
			Heterogeneous	Relatively homogeneous	Two main types

theroid bacilli occur. In one series, for example, only 10 per cent of the strains typed as *gravis* were toxigenic. Furthermore, a certain proportion of toxigenic strains cannot be allocated into one or another of these types. The proportion of indeterminate strains is said to be higher when diphtheria is mild. Anderson et al. found about 5 per cent of their strains (British) could not be typed, and 44 per cent of the strains found by Seligmann[67] in New York City in 1940 were indeterminate.

Pathogenicity for Man.[22] Diphtheria is primarily a disease of childhood, and the age incidence is an expression of waning passive immunity of maternal origin and the development of an active immunity on the one hand, and the risk of exposure on the other. The very young child is passively protected and not exposed to great risk of infection, but by school age the immunity has disappeared in large part and risk of exposure to infection is tremendously increased with entrance into school. The adolescent and adult have acquired an active immunity as a consequence of clinical or, more commonly, inapparent infection. Thus clinical diphtheria is most common in the 5 to 14 age group, though the carrier state and inapparent infection are probably no more so than in the higher age groups. Though diphtheria is in large part preventable by artificial immunization, particularly of the preschool child, it continues to be an important disease.

Diphtheria in man is usually a local infection of the mucous surfaces. The pharynx is most commonly affected, but infection of the larynx, or membranous croup, and nasal diphtheria, or membranous rhinitis, are not infrequently observed. Diphtheritic infections of the conjunctiva and of the middle ear are less common, and cutaneous or wound diphtheria is only occasionally observed. The last, however, may assume considerable proportions under certain circumstances. Ulcerative diphtheria of the skin, sometimes called desert sore or tropical ulcer, has been observed in epidemic form in Haifa, and ulcers of the deep, punched-out type occurred with some frequency in troops living under combat conditions in the south and central Pacific areas during World War II.[2, 36] Infection of the mucous surfaces of the genital organs is occasionally found. The invasion of other localities is rare; primary infection of the lungs and diphtheritic meningitis have been observed, and infection of the umbilicus in the newborn has been reported.

It has long been known that diphtheria bacilli are not often found in the internal organs, but the frequency with which such infection occurs is not definitely known. Diphtheria bacillus septicemia is occasionally observed, and the suggestion frequently occurs in the German literature that generalized infection with the diphtheria bacillus occurs more often than is generally believed.[73] A few cases of acute vegetative endocarditis caused by the diphtheria bacillus have been reported.[56]

The chief local consequence of infection is a degeneration of the epithelial cells, extending to the underlying tissues and accompanied by a profuse fibrinous exudation, and the characteristic diphtheritic membrane, containing fibrin, dead tissue cells, leucocytes, and bacteria, is formed on the affected surface. The mechanical interference of the membrane with breathing may assume significant proportions and even necessitate intubation or tracheotomy.

Although diphtheria toxin undoubtedly plays a part in the formation of the membrane, its systemic effects following absorption are by far the most important, and diphtheria is, like tetanus, essentially a toxemia. The organs most severely affected are the kidneys, heart, and nerves. A variety of lesions may be found in the kidneys, acute interstitial nephritis being the most common. The lesions in the heart consist commonly of a fatty degeneration in the muscle fibers, which may be very extensive. The diphtheritic myocarditis occurring as a complication usually leaves no permanent damage, but this is not always true.[9, 66] Fatty degeneration also occurs both in the myelin sheath of the peripheral nerves and in the white matter of the brain and cord.

These changes in muscle and nerve account for the serious cardiac weakness often observed in diphtheria and the frequent occurrence of the more or less extensive paralysis which so commonly follows an attack of the disease. It is probable that a small amount of toxin can cause extensive damage in these tissues.

Pathogenicity for Lower Animals. Diphtheria is not a natural disease of lower animals. There is a popular belief that cats may become infected and disseminate the bacilli, but this is not true. Both the local and general symptoms of human diphtheria in man can, however, be reproduced by animal inoculation. Inoculations upon the healthy mucous membrane of most adult animals lead to no changes, but if young animals are injected intratracheally, or if the mucous surface is injured before inoculation, a characteristic false membrane is produced which is histologically identical with that found in man.

The subcutaneous inoculation of a guinea pig with a sufficient amount of a young broth culture or toxic filtrate will produce death in 1 to 4 days, the time depending upon the size of the inoculum. The animal becomes obviously ill 12 to 18 hours after inoculation, and nephritic symptoms, paralytic manifestations, and other characteristics of human diphtheria are often observed. Postmortem findings include edema and possibly necrosis at the site of inoculation, congestion of the regional lymphatics and abdominal viscera, a pleural exudate, and, characteristic of diphtheritic toxemia in this animal, an enlarged and hemorrhagic condition of the adrenals. As a rule, the bacilli remain localized and are not found in large numbers in the internal organs of the infected animal. Guinea pigs that receive smaller doses and do not die by the fourth day may develop paralytic symptoms and cachexia and die later on, a condition obviously different from the acute toxemia.

Animals vary considerably in their susceptibility to infection. Rats and mice are relatively refractory; rabbits are less susceptible than guinea pigs; cats, dogs, young chicks and pigeons are highly susceptible. Paralytic manifestations appear more frequently in dogs and pigeons than in guinea pigs or rabbits and may be produced in the relatively refractory rat.[1]

Toxin Assay. Assay of toxicity is most commonly carried out in the guinea pig[27] and by intradermal inoculation in the rabbit (see below); the latter is considerably more sensitive and dependent upon a local rather than a lethal response.[20] The baby chick is uniformly susceptible to the toxin, dying within 24 hours, and its use has been urged by a number of workers.[10, 18, 38] The toxin is also active against various kinds of cells in culture, and tissue cultures may be used to assay the activity.[41, 57, 68] Any such assay may be made use of in the virulence test for toxigenicity and/or applied to the titration of antitoxin.

Bacteriological Diagnosis. To establish a diagnosis of infection with diphtheria bacilli, in either case or carrier, the bacillus must be isolated and its toxigenicity demonstrated. The specimen is taken on a swab, either plain or previously dipped in sterile horse serum which is coagulated on the surface by twirling in a flame. It is best to inoculate two mediums, Löffler's serum agar and a tellurite medium such as chocolate tellurite agar; if only a single medium can be used, tellurite is preferable. A blood agar plate should be inoculated as well, both for the isolation of diphtheria-like colonies and to provide for the cultivation of hemolytic streptococci which may be present. After the plates have been inoculated a smear may be made by rolling the swab on a slide, and stained with alkaline methylene blue; it will serve to show the presence of the spirochetes and fusiform bacilli of Vincent's angina should these be present.

Diphtheria bacilli grow up in 18 to 24 hours' incubation. If the characteristic black or gray colonies appear on tellurite, smears may be made from such colonies and from the Löffler slant for microscopic examination; the morphology of the diphtheria bacillus is fre-

quently not characteristic on tellurite as indicated above. It is often inferred that only diphtheria bacilli grow as black colonies on tellurite medium. This is not true, for any bacterium that reduces tellurite will produce similar colonies; tellurite-reducing bacteria, other than diphtheria bacilli, from the nose and throat are usually staphylococci or micrococci, and as a rule their colonies resemble those of the *mitis* variety of diphtheria bacillus but are blacker.

The virulence test. If morphologically typical bacilli are found, toxigenicity must be tested by animal inoculation. This is ordinarily carried out in the guinea pig by subcutaneous or intracutaneous inoculation. In the first instance the growth from a Löffler slant is suspended in 10 ml. saline and 4 ml. injected subcutaneously into each of two guinea pigs, one of which has received 250 units of diphtheria antitoxin 24 hours previously. The diphtheria bacillus will kill the unprotected pig in 3 to 5 days, and autopsy will show local edema and the characteristic hemorrhagic enlarged adrenals, while the protected animal will survive. For the intracutaneous test the growth from a Löffler slant is suspended in 20 ml. saline and 0.15 ml. injected into the shaved abdominal skin of each of two pigs as above. Toxigenicity is indicated by the development of a local infiltrated lesion which shows superficial necrosis in 2 or 3 days in the unprotected pig. By the latter technique a number of tests may be carried out in the same pair of animals.

The virulence test may also be carried out in the rabbit. The growth from a Löffler slant culture is suspended in 2 to 3 ml. of sterile infusion broth, and 0.1 ml. injected intradermally. Four hours later the animal is given 1000 units of antitoxin intravenously, and immediately afterwards a second intradermal inoculation of 0.1 ml. of the bacterial suspension is made at a site adjacent to that of the first inoculation. Reactions should be read at 72 hours. If the strain of bacteria is toxigenic, the site of the first inoculation will be a central necrotic area, usually hemorrhagic, surrounded by a zone of erythema. The inoculation of antitoxin does not affect a reaction to the first inoculation, but does specifically inhibit a reaction to the second inoculation, and the site of the latter appears as a small, pinkish papule. Eight to ten such virulence tests may be carried out simultaneously in the same animal.

As indicated above, toxigenicity, or virulence, may also be assayed in other animals, but such virulence tests have not been generally used. An *in vitro* virulence test based on the gel-diffusion technique has been widely applied. It consists essentially in growth of the bacterial strain under consideration on an agar plate in which antitoxin has been placed in a well or trench in the agar medium. The toxin diffusing from the growth forms a line of specific precipitate with the antitoxin diffusing from the reservoir.[7, 21, 26, 53] It is necessary to include a known toxigenic strain for control purposes.

Immunity. Immunity to diphtheria resulting from recovery from a frank attack of the disease, a subclinical infection, or prophylactic inoculation, is essentially an antitoxic immunity, *i.e.*, an immunity to the disease rather than the infection. Although the toxin is of overriding importance, there is evidence that other toxic substances formed by diphtheria bacilli may contribute to the pathogenesis of the disease,[11] and the question of the significance of an antibacterial immunity is one of perennial interest.[8, 24, 40] The latter is complicated by the serological heterogeneity of the diphtheria bacilli noted above. Effective immunity is, however, generally considered to be primarily antitoxic in nature.

Titration of antitoxin. Assay of antitoxin is based on the demonstration of its specific reaction with a standardized toxin and is, in essence, a quantitation of proof of toxin specificity in the several kinds of virulence tests. The classic methods of assay in the guinea pig, described elsewhere (Chap. 14), permit the quantitation of antitoxic activity on the basis of an arbitrarily defined unit. This is necessarily a reflection of a defined

dose limit of toxin, the L_+ dose, based on death of the guinea pig, and the Lr, or reactive, dose determined by intradermal titration. Since the toxin consists of both toxin and toxoid, a standard antitoxin must be used to define a standard toxin reagent, and the toxin so standardized is, in turn, used to assay the unknown antitoxin in units of activity.

The proportions of toxin and toxoid in a given preparation do not affect its specific reaction with antitoxin *in vitro,* and either toxin or antitoxin may be titrated against a standard by specific precipitation in the flocculation test. This consists of a determination of the zone of optimal proportions, and the diphtheria toxin–horse antitoxin is the classic system in the so-called H type of flocculation reaction (Chap. 14). In practice flocculation, or precipitation, occurs most rapidly and in largest amount when the toxin and antitoxin are present in optimal proportions, and assay of the flocculation, or Lf, dose is readily carried out. In the standardization of commercial antitoxin, the flocculation titration is usually carried out to facilitate the subsequent guinea pig assay required by law.

The flocculation reaction is not sufficiently sensitive to measure the very small amounts of antitoxin demonstrable by the intradermal method, and there has been some interest in the use of passive hemagglutination as a sensitive *in vitro* method. Toxin, but apparently not toxoid, is adsorbed on tannic acid–treated red cells, and these are specifically agglutinated in the presence of antitoxin. It has been reported[23] that less than 0.01 unit of antitoxin may be measured in this way.

The Schick test. Immunity to diphtheria, then, may be measured by the amount of circulating antitoxin present in a given individual. A skin test has been devised by Schick, and is known as the *Schick test,* in which a minute amount of diphtheria toxin is injected intradermally. In the nonimmune the irritant action of the toxin gives rise to local erythema followed by necrosis and desquamation, and the reaction is said to be positive. In the immune, however, the toxin is neutralized by the antitoxin that is present, the characteristic reaction does not develop, and the reaction is negative. The amount of toxin injected is usually 1/50 of a guinea-pig MLD in a volume of 0.1 or 0.2 ml.; the Permanent Standards Committee of the League of Nations specifies 1/40 MLD in 0.2 ml. and 1/50 MLD in 0.1 ml. According to Taylor and Moloney,[12, 71] however, a toxin of three times this strength gives more sharply defined reactions. Considerable interest has attached to diluents for the Schick toxin since it is not stable in phenol-saline solutions. The dilute toxin is, however, stable in 2 per cent peptone solution, a borate buffer–gelatin solution, and a glycerol-gelatin solution which has been proposed recently. The advantages of a ready-diluted Schick toxin are obvious, and toxins diluted ready for use are now generally available.

For many years a negative Schick test has been regarded as indicating the presence of 1/20 unit or more of antitoxin per ml. in the blood serum and a positive test less than 1/40 unit. More recent experiments, however, have indicated that the so-called Schick level of immunity is much lower than this and in the neighborhood of 1/250 to 1/500 unit of antitoxin; negative reactions have been obtained in persons with as little as 0.0005 unit. A number of workers are of the opinion that a negative Schick test not only is indicative of antitoxin content of the blood but also involves a defense mechanism other than that of antitoxin production.

A scarification test in which diphtheria toxin is introduced by punctate scarification rather than intradermal injection has been introduced by Reh and is called *Reh's test.* It is said to be somewhat simpler to perform than the Schick test and, when carried out with a potent toxin (with a guinea-pig MLD of 2000 per ml.), to give parallel results with the Schick test.

The question of whether the Schick test is indicative of a degree of immunity such that subsequent infection is highly

improbable is one that cannot be answered a priori. Experience has shown, however, that the assumption that a Schick-negative person is, for all practical purposes, immune, is pragmatically sound.

Prophylactic Immunization.[37, 70] It was early observed that experimental animals can be immunized to diphtheria by the injection of living cultures of the bacilli after a protective dose of antitoxic serum or by the inoculation of toxin neutralized with antitoxin. Theobald Smith suggested the use of toxin-antitoxin mixtures in the immunization of horses in 1907, and the same method was used by von Behring in 1913 to immunize children. The use of toxin-antitoxin for the immunization of man, however, was developed largely through the efforts of Park in New York City from 1913 onward.

Toxin-antitoxin. The mixture usually used contains 0.1 L_+ dose of toxin per ml. The toxin is slightly underneutralized (5 ml. of the mixture should produce diphtheritic paralysis in 300 gm. guinea pigs) but depends for its immunizing efficiency not on the slight excess of toxin but on a slow dissociation of the toxin-antitoxin complex to liberate free toxin. Administered in three doses of 1 ml. each at intervals of 1 to 2 weeks, toxin-antitoxin produces an immunity in 85 per cent of individuals inoculated. The immunity develops slowly, and 1 to 6 months may be required for the Schick reaction to become negative. Accidents may occur as a consequence of dissociation of the toxin-antitoxin mixture—freezing in one instance produced such dissociation—but these are rare, particularly with the 0.1 L_+ dose mixture. There is the possibility of sensitization of the inoculated individual to horse serum.

Toxoid. The use of formol toxoid or anatoxin as an immunizing agent was introduced by Ramon in 1923 and has been widely adopted. As pointed out elsewhere (Chap. 9), toxin treated with formaldehyde (in this case a potent toxin of more than 15 Lf doses per ml. is incubated with 0.3 to 0.4 per cent formalin at 37° C. for 1 month) loses its toxicity but retains its antigenicity and is a highly efficient immunizing agent. The administration of this material in three doses of 0.5, 1.0, and 1.0 ml. at intervals of 2 to 3 weeks renders 95 per cent of persons Schick-negative. It was at first thought that toxoid might entirely replace toxin-antitoxin as an immunizing agent, but this has not proved to be the case. Reactions to the bacillary protein, while not of great importance as a rule in young children, may be relatively severe in older persons and its use is preferably restricted to children under 12 years of age. Reactivity may be tested for by the intradermal injection of toxoid—the *Moloney test.*

Toxoid-antitoxin floccules (the precipitate coming down at the optimal antigen-antibody ratio) have been used in England to a considerable extent. There is, presumably, a partial purification of the toxoid by precipitation with antibody. This material and toxin-antitoxin floccules have not been widely used in the United States. Toxoid precipitated with protamine appears to be an effective immunizing agent without giving the untoward reactions sometimes observed with alum-precipitated toxoid. Another kind of preparation that has been used in England is toxoid adsorbed on aluminum phosphate, diphtheria phosphate toxoid or DPT.

Alum-precipitated toxoid. It has been found that toxoid precipitated with potassium alum (small amounts, 1 to 2 per cent, are required) is superior as an immunizing agent to ordinary formol toxoid. Present preparations are treated with charcoal prior to alum precipitation to remove color and extraneous nitrogenous material. The precipitate is insoluble (it may be redissolved in sodium citrate or sodium tartrate) and remains in the subcutaneous tissue for a considerable time, thus providing a prolonged antigenic stimulus. It was first thought that a single injection of this material was sufficient to provide a solid immunity; some of the early reports indicated that 90 to 95 per cent of Schick-positives became Schick-negative as a consequence of a single injection. The

administrative advantages of a single injection are, of course, obvious. It subsequently became clear that a single injection is not sufficient; as little as 11 per cent conversion has been reported. There is also a tendency for such Schick-negatives to revert to Schick-positives within a year or two, though why this should be the case is not at all clear. It seems established that a single injection is not sufficient but two injections provide a solid immunity. The primary dose should be not less than half the total toxoid given and may be as great as two-thirds of it. Alum toxoid has the same tendencies to produce untoward reactions in older persons that are observed with formol toxoid.

Passive Immunity. Susceptible, *i.e.*, Schick-positive, individuals may be passively immunized to diphtheria by the injection of antitoxic horse serum or purified preparations of antitoxin. Such immunity is of relatively short duration and is not effective for longer than 2 or 3 weeks at the most. Passive immunization is not so extensively practiced now as it formerly was but is indicated in the case of susceptible individuals who are directly exposed to the disease. Except on a small scale, as in a hospital ward, it is not practical to control epidemic diphtheria through passive immunization.

The use of combined active-passive immunization in which both toxoid and antitoxin are given simultaneously, the former in protective amounts, has been of some interest. Passive protection conferred by antitoxin seems not to interfere seriously with the immune response though there is a period of low immunity, after the second or third week, after the passive protection has been exhausted. A second inoculation of toxoid is highly desirable.

The therapeutic use of antitoxin. Serum therapy is more successful in diphtheria than in any other disease, and there is no question of its efficacy in reducing the case fatality rates. As in the case of tetanus and botulism, the therapeutic administration of antitoxin cannot bring about repair of tissues already damaged by toxin. Early administration is, therefore, essential, and there is progressive increase in the case fatality rate with each day's delay. There is no limit, beyond the volume, to the number of units that may be safely injected. Antitoxin is generally administered intramuscularly but in severe cases may be given intravenously. It is completely ineffective when given by mouth.

Usually horse serum contains 500 to 700 units per ml. and exceptionally 1000 to 1500. Concentration of the antitoxin by salting out and other procedures is generally practiced, for, although some antitoxin is lost in the process, the concentration is increased with a corresponding reduction in the volume to be injected.

Epidemiology.[65] The epidemiology of diphtheria is considerably better understood than that of any other disease, in part because the causative agent can be isolated with relative facility from infected individuals, and in part because the Schick test allows the differentiation of the immunes and the nonimmunes. As in the case of other respiratory diseases, infectious material leaves the body in the secretions of the nose and throat, is transmitted from man to man by contact or infective droplets, and enters the body via the mouth and nose. Furthermore, the diphtheria bacillus is disseminated not only by persons with the disease but also through the agency of healthy carriers in whom there is no clinical evidence of infection. Unlike many of the diseases of the respiratory tract, however, diphtheria is an immunizing disease, and prolonged or repeated contact with the bacillus frequently results in the development of a solid immunity to the disease in its clinical manifestations.

Immunity and susceptibility. Schick testing indicates that while susceptibility is low in the first 6 months of life, the proportion of Schick-positives increases rapidly and is at a maximum in children under 4 or 5 years of age, then gradually declines. The initial immunity of the very young is passively transferred from the mother and is not of long duration. The increase in the proportion of immunes,

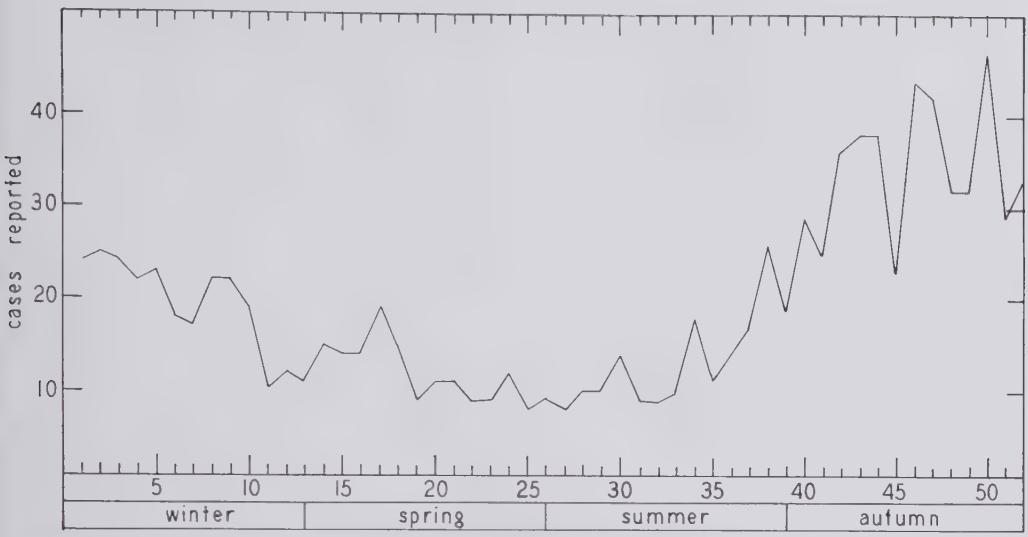


Figure 174. The seasonal incidence of diphtheria shown by the 5-year median number of cases reported weekly for the period 1956–60. Data from the Morbidity and Mortality Weekly Report, vol. 9, 1961. Communicable Disease Center, Public Health Service, U. S. Department of Health, Education and Welfare.

however, is by no means entirely a result of recovery from clinical diphtheria, and the question arises as to how these individuals acquire an active immunity.

Carriers. As indicated above, healthy individuals may harbor virulent diphtheria bacilli in their throats. These carriers need be neither immunes nor convalescents and are, for the most part, casual carriers. There is no precise information concerning the duration of this transient carrier state; it may possibly be about 2 weeks. The proportion of carriers has been investigated by a number of workers. In a study of Baltimore school children, Doull and Fales found an average carrier rate of 2.32 per cent from November to May. On the basis of this Frost has estimated the carrier incidence in the 5 to 14 age group in that city to be 2538 per 10,000. At this rate 75 per cent of the population becomes infected at least once in 5 years, 95 per cent in 10 years, and over 99 per cent in 15 years, while very considerable proportions would suffer repeated infections, the average being 2.5 infections per person in 10 years. Others have recorded considerably higher carrier rates; Dudley has reported 6.6 per cent in a boys' school, and repeated swabbings showed that at least 40 per cent carried the diphtheria bacillus at one time or another during the yearly period.

There is, it appears, ample opportunity for contact with virulent diphtheria bacilli, and there is every reason to suppose that the increasing proportion of Schick-negatives in the progressively higher age groups is a consequence of an active immune response to the presence of these microorganisms in the nose and throat.

The Control of Diphtheria. It will be obvious from the above considerations that diphtheria is widely disseminated in the human population and cannot be controlled by the isolation of carriers or, except in a strictly limited sense, by quarantine of cases. The diphtheria bacillus is especially sensitive to erythromycin, which has been used with some success in treating carriers.[6, 74]

The control of diphtheria is entirely a matter of immunization and, if a sufficiently large proportion of the susceptible population is rendered immune, the prevalence of clinical diphtheria should decrease. Godfrey found that the immunization of 50 per cent or more of the children of school age, 5 to 14 years of age, did not produce a fall in the incidence of diphtheria in a number of large American cities, but that when 30 per cent or over of the preschool children

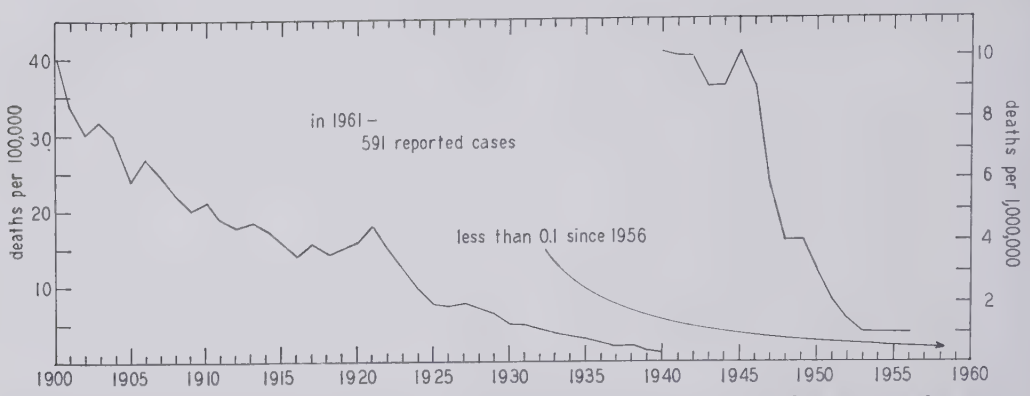


Figure 175. The prevalence of diphtheria since 1900 as shown by the mortality rates in the Registration Area. Data from the Morbidity and Mortality Weekly Report, vol. 9, 1961. Communicable Disease Center, Public Health Service, U. S. Department of Health, Education and Welfare.

were immunized there was a definite reduction in the incidence of diphtheria not only among these children but in the community as a whole. It is commonly assumed that immunization of 70 per cent of children suffices to control epidemic diphtheria. Other as yet unknown factors are involved also, for the disease persists in relatively high incidence in some well-immunized communities or groups.[48, 52] Immunization confers a marked, though not absolute, protection in the individual case, and it would appear best from both the individual and group points of view that immunization be effected early.

To what extent prophylactic inoculation and the therapeutic use of antitoxin have influenced the decline in diphtheria is problematical. The disease was endemic during the first half of the nineteenth century though showing increasing epidemic tendencies. Between 1850 and 1860 a great pandemic developed, apparently from a focus in France, which swept over the world. A high mortality was maintained for 25 or 30 years, then around 1885 a decline set in which continued to about 1941. Immunization was not generally practiced until about 1920, though antitoxin therapy began somewhat earlier.

Beginning in 1941 there was a general increase all over the world in the prevalence of diphtheria. It apparently began in Germany in 1939, possibly due in part to mass movement of children into camps without adequate immunization, with a doubling of the already high (285 and 207 in Austria and Germany) morbidity rate, and an increase in severity as indicated by increase in case fatality rates of 3.8 per cent in 1937–38, to 4.4 per cent in 1939, and 5.0 per cent in 1940. The disease spread into neighboring countries in northwest Europe. In Belgium the number of cases rose from 2419 in 1939 to 16,072 in 1943; in Holland there were 1273 cases in 1939, 5501 in 1941, 19,527 in 1942, and 56,603 in 1943; in Norway there were 54 cases in 1939 and 22,787 in 1943. Diphtheria was the leading epidemic disease of the war years in Europe and an important cause of death in the Germany army. The rates have subsided from these highs, but diphtheria continues to be more prevalent in Europe than elsewhere in the world.[64]

These rates were reflected in U. S. military personnel stationed in Europe; diphtheria was a major problem with the incidence six times greater than for comparable troops stationed in the United States. Since 1946 the rate has declined. The number of cases and deaths in 1945 exceeded those of the previous 5 years, but the case fatality rate was not appreciably altered.[49] In the United States there was a slight rise in the incidence of diphtheria, but it has subsequently declined; in 1960, 918 cases were reported in contrast to 17,987 in 1941. The decline has been in infants and children for the most part, but since 1940 there has been a slow but steady increase in the age

groups over age 10,[72] possibly a reflection of failure to re-immunize in the higher age groups. The residual infection tends to be concentrated in the nonwhite segment of the population, and further reduction would appear to be, in part, a matter of immunization of this group.[54]

THE DIPHTHEROID BACILLI

Microorganisms morphologically closely similar to and frequently indistinguishable from the diphtheria bacillus, known as diphtheroid bacilli, are found in man and lower animals. Three species are commonly observed in man. A form occurring in the human throat and readily confused with the diphtheria bacillus on microscopic examination was first observed by Löffler and by von Hofmann-Wellenhof. It is known as *Hofmann's bacillus, Corynebacterium hofmannii* or *C. pseudodiphtheriticum.* It differs slightly from the diphtheria bacillus in that it is somewhat shorter and plumper and does not ferment dextrose. Most important, it does not form a soluble toxin and is readily differentiable from *C. diphtheriae* by the virulence test. It seems to be completely nonpathogenic for man and experimental animals.

A second species, *C. xerose,* has been isolated repeatedly from a form of conjunctivitis known as xerosis, but its etiological relation to the disease is uncertain. It is also found on the skin where it is presumably a part of the normal bacterial flora, and it is probable that its presence in xerosis is that of a contaminant. It does not form a soluble toxin. *C. acnes* has been found in acne pustules, but whether the association is causal is open to question. This organism stands somewhat apart from the other bacilli of the group in that it is microaerophilic and grows profusely under anaerobic conditions with the formation of a pink pigment. It also does not form a soluble toxin.

A number of species of corynebacteria are pathogenic for lower animals and rarely may infect man. *C. pyogenes* is one of the commonest causes of purulent infections in cattle, sheep, pigs, and goats; it is the cause of a form of mastitis, a few cases of abortion, arthritis, and granulomatous lesions in bovines, and is associated with calf pneumonia as well. Infections of man have been reported.[3] It forms a soluble toxin, immunologically distinct from and considerably weaker than that of the diphtheria bacillus, which is hemolytic for rabbit erythrocytes, lethal for mice, and produces dermal necrosis in the rabbit similar to that produced by diphtheria bacillus toxin. This bacterium and its toxin have been studied extensively by Lovell.[42, 43, 44, 45, 46]

C. ovis (*C. pseudotuberculosis*) or the Preisz-Nocard bacillus is also a pathogen

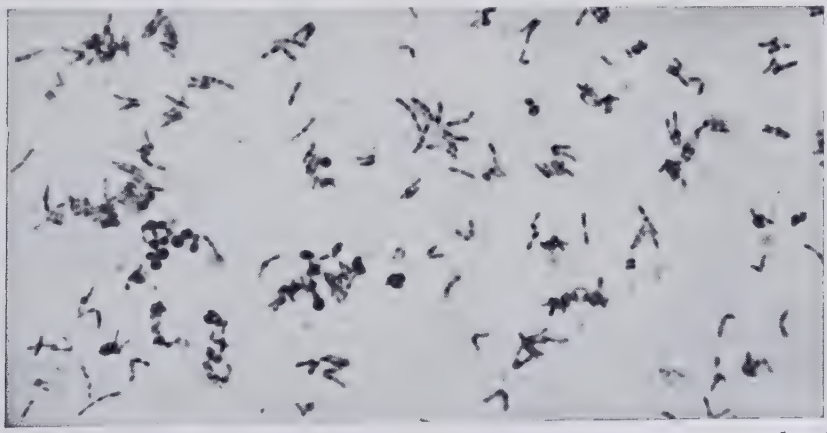


Figure 176. *Corynebacterium pseudodiphtheriticum.* Smear from pure culture stained with alkaline methylene blue. Note the irregular staining, club-shaped forms, and general close resemblance to *C. diphtheriae.* × 1050.

of domestic animals. It produces a caseous lymphadenitis and ulcerative lymphangitis in sheep and horses referred to as pseudotuberculosis, and ulcerative lesions in other domestic animals. Like *C. pyogenes*, it forms a weak exotoxin distinct from diphtheria toxin. *C. renale* is closely related serologically to *C. ovis* and produces purulent infections of the urinary tract in cattle, sheep, horses, and dogs. *C. equi* is the cause of spontaneous pneumonia in foals and other infections in horses; this species is of interest in that it is variable in its reaction to the acid-fast stain, the coccoid forms retaining the stain while the bacillary forms take the counterstain, suggesting a relationship to the mycobacteria and acid-fast actinomycetes. *C. enzymicum* has been isolated from man primarily, but has been found as the cause of an epidemic ophthalmia of sheep. *C. murisepticum* is the cause of mouse septicemia and is apparently pathogenic for no other animals. The occurrence of these and other diphtheroid bacilli in diseases of domestic animals makes this group of microorganisms of considerable interest in veterinary medicine.

In addition to these a dozen or more authenticated species of corynebacteria are soil saprophytes or pathogenic for plants, producing diseases of wheat, alfalfa, ring rot of potato, bacterial canker of tomato and poinsettia, bean wilt, and the like.

REFERENCES

1. Agarwal, S. C., and Pryce, D. M.: Experimental diphtheritic paralysis in rats. J. Path. Bact., 1959, **78:**171–177.
2. Bacon, D. F., and Marples, M. J.: Researches in Western Samoa. II. Lesions of the skin and their bacteriology. Trans. Roy. Soc. Trop. Med. Hyg., 1955, **49:**76–81.
3. Ballard, D. O., Upsher, A. E., and Seely, D. D.: Infection with *Corynebacterium pyogenes* in man. Amer. J. Clin. Path., 1947, **17:**209–215.
4. Barksdale, W. L., Garmise, L., and Horibata, K.: Virulence toxinogeny, and lysogeny in *Corynebacterium diphtheriae*. Ann. N. Y. Acad. Sci., 1960, **88:**1093–1108.
5. Barksdale, W. L., Garmise, L., and Rivera, R.: Toxinogeny in *Corynebacterium diphtheriae*. J. Bact., 1961, **81:**527–540.
6. Beach, M. W., Gamble, W. B., Zemp, C. H., Jr., and Jenkins, M. Q.: Erythromycin in the treatment of diphtheria and diphtheria carrier state. Pediatrics, 1955, **16:**335–344.
7. Bodily, H. L., Cuculich, T. F., and Benjamin, F.: Application of in vitro virulence test for *Corynebacterium diphtheriae* in public health laboratory. Amer. J. Pub. Health, 1952, **42:** 246–248.
8. Bowen, H. E., Wyman, L., and McComb, J. A.: Cellular vaccines and toxoid in the immunization of animals against diphtheria. Amer. J. Hyg., 1954, **59:**306–317.
9. Boyer, N. H., and Weinstein, L.: Diphtheritic myocarditis. New England J. Med., 1948, **239:** 913–919.
10. Branham, S. E., Grabowski, M. W., and Riggs, D. B.: Evaluation of the chick as a test animal in the assay of diphtheria toxoids. Appl. Microbiol., 1957, **5:**286–291.
11. Branham, S. E., Hiatt, C. W., Cooper, A. D., and Riggs, D. B.: Antigens associated with the toxin of the *gravis* type of *Corynebacterium diphtheriae*. J. Immunol., 1959, **82:**397–408.
12. Cameron, G. D. W., and Gibbard, J.: Report of a trial of a new Schick-toxin. J. Immunol., 1941, **40:**47–51.
13. Christensen, P. E.: Studies on lysogenicity in *C. diphtheriae*. Acta Path. Microbiol. Scand., 1957, **41:**67–78.
14. Christensen, W. B.: Observations on the staining of *Corynebacterium diphtheriae*. Stain Technol., 1949, **24:**165–170.
15. Ciuca, M., et al.: Potentiel de "variation" et capacité d'adaptation entrecroisée des bactériphages—antistaphylococciques et antidiphtériques—sur des souches homologues ou hétérologues, soumises à la "lysogénéisation." Arch. Roumaines Path. Exper. Microbiol., 1958, **17:**595–612.
16. Cuica, M., et al.: Rôle des bactériphages dans l'écologie microbienne. Note I. Le complexe écologique de la microflore dans le rhinopharynx des malades, par rapport à l'évolution et aux particularités cliniques de l'angine diphtérique. Arch. Roumaines Path. Exper. Microbiol., 1959, **18:**493–507.
17. Cuica, M., et al.: Rôle des bactériphages dans l'écologie microbienne. Note II. Transduction de la lysogénie-homo-hétérologue diphtérostaphylococcique, dans les conditions écologiques naturelles de l'angine diphtérique. Arch. Roumaines Path. Exper. Microbiol., 1959, **18:**509–518.
18. Dishon, T.: The susceptibility of the developing chick embryo to diphtheria toxin and its use in the titration of antitoxin. Bull. Res. Council Israel, 1957, **6E:**97–103.
19. Edwards, D. C.: The growth and toxin production of *Corynebacterium diphtheriae* in submerged culture. J. Gen. Microbiol., 1960, **22:** 698–704.
20. Eisler, M.: Das letale und nekrotisierende Gift in Diphtherie-Bouillonkulturen. Ztschr. f. Immunitätsf. u. Exper. Therap., 1958, **115:**215–223.
21. Elek, S. D.: The plate virulence test for diphtheria. J. Clin. Path., 1949, **2:**250–258.
22. Fisher, A. M., and Cobb, S.: The clinical manifestations of the severe form of diphtheria. Bull. Johns Hopkins Hosp., 1948, **83:**297–325.
23. Fisher, S.: The estimation in vitro of small amounts of diphtheria antitoxin by means of a haemagglutination technic. J. Hyg., 1952, **50:**445–456.
24. Fleck, L., and Kunika, A.: The significance of

antibacterial immunity in diphtheria. Texas Repts. Biol. Med., 1957, **15**:850–860.
25. Freeman, V. J.: Studies on the virulence of bacteriophage infected strains of *C. diphtheriae*. J. Bact., 1951, **61**:675–688.
26. Frobisher, M., Jr., King, E. O., and Parsons, E. I.: A test in vitro for virulence of *Corynebacterium diphtheriae*. Amer. J. Clin. Path., 1951, **21**:282–285.
27. Gerwing, J., Long, D. A., and Mussett, M. V.: The assay of diphtheria toxin. Bull. W. H. O., 1957, **17**:537–551.
28. Groman, N. B.: Evidence for the active role of bacteriophage in the conversion of nontoxigenic *Corynebacterium diphtheriae* to toxin production. J. Bact., 1955, **69**:9–15.
29. Groman, N. B., and Eaton, M. J.: Genetic factors in *Corynebacterium diphtheriae* conversion. J. Bact., 1955, **70**:637–640.
30. Groman, N. B., Eaton, M., and Booher, Z. K.: Studies of mono- and polylysogenic *Corynebacterium diphtheriae*. J. Bact., 1958, **75**:320–325.
31. Gundersen, W. B., and Henriksen, S. D.: Conversion in *Corynebacterium belfanti* by means of a temperate bacteriophage originating from a toxigenic strain of *Corynebacterium diphtheriae*. Acta Path. Microbiol. Scand., 1959, **47**:173–181.
32. Hatano, M., Nakamura, K., and Kurokawa, M.: Isolation of a new temperate phage causing the lysogenic conversion in *Corynebacterium diphtheriae*. Japanese J. Microbiol., 1959, **3**: 301–311.
33. Hermann, G. J., and Parsons, E. I.: A study of antigenic relationships in some strains of *Corynebacterium diphtheriae*. Amer. J. Hyg., 1955, **61**:64–71.
34. Hewitt, L. F.: Cell structure of *Corynebacterium diphtheriae*. J. Gen. Microbiol., 1951, **5**:287–292.
35. Hewitt, L. F.: Diphtheria bacteriophages and their relation to the development of bacterial variants. J. Gen. Microbiol., 1952, **7**:362–371.
36. Hollander, M. H.: Diphtheria of the skin. U. S. Armed Forces Med. J., 1951, **2**:229–232.
37. Holt, L. B.: Developments in Diphtheria Prophylaxis. Heineman Medical Books, Ltd., London. 1950.
38. Kolb, R. W., Branham, S. E., and Riggs, D. B.: Comparison of the guinea pig and the chick in evaluation of diphtheria toxin for the Schick test. Appl. Microbiol., 1955, **3**:242–247.
39. Kröger, E., Thofern, E., and Schulz, K.: Serologische Untersuchungen bei Diphtheriebakterien. Ztschr. f. Hyg. u. Infektionskr., 1952, **134**:474–487; **135**:254–274.
40. Lautrop, H.: On the existence of an antibacterial factor in diphtheria immunity. Acta Path. Microbiol. Scand., 1955, **36**:274–288.
41. Lennox, E. S., and Kaplan, A. S.: Action of diphtheria toxin on cells cultivated in vitro. Proc. Soc. Exptl. Biol. Med., 1957, **95**:700–702.
42. Lovell, R.: Studies on *Corynebacterium pyogenes* with special reference to toxin production. J. Path. Bact., 1937, **45**:339–355.
43. Lovell, R.: The *Corynebacterium pyogenes* antitoxin content of animal sera. J. Path. Bact., 1939, **49**:329–338.
44. Lovell, R.: Studies on the toxin of *Corynebacterium pyogenes*. J. Path. Bact., 1941, **52**:295–303.
45. Lovell, R.: Further studies on the toxin of *Corynebacterium pyogenes*. J. Path. Bact., 1944, **56**:525–529.
46. Lovell, R.: *Corynebacterium pyogenes* infections of domestic animals. Vet. Record, 1945, **57**: 383–385.
47. McLeod, J. W.: The types *mitis, intermedius* and *gravis* of *Corynebacterium diphtheriae*. Bact. Rev., 1943, **7**:1–41.
48. Moore, H. A.: Diphtheria in the United States in 1956. Pub. Health Repts., 1958, **73**:439–444.
49. Moore, H. A., and Larsen, G. I.: Present distribution of diphtheria in the United States. Pub. Health Repts., 1957, **72**:537–542.
50. Morton, H. E.: *Corynebacterium diphtheriae*. A correlation of recorded variations within the species. Bact. Rev., 1940, **4**:177–226.
51. Mueller, J. H.: Nutrition of the diphtheria bacillus. Bact. Rev., 1940, **4**:97–134.
52. Murphey, W. J., Maley, V. H., and Dick, L.: Continued high incidence of diphtheria in a well-immunized community. Pub. Health Repts., 1956, **71**:481–486.
53. Ouchterlony, O.: In vitro method for testing the toxin-producing capacity of diphtheria bacteria. Acta Path. Microbiol. Scand., 1949, **26**: 516–524.
54. Page, M. I.: The present problem of diphtheria control in the United States. Amer. J. Pub. Health, 1962, **52**:68–74.
55. Pappenheimer, A. M.: The pathogenesis of diphtheria. Society for General Microbiology, Symposium No. 5, pp. 40–56. Cambridge University Press, 1955.
56. Pike, C.: Corynebacterial endocarditis: With report of a case due to toxigenic *Corynebacterium diphtheriae*. J. Path. Bact., 1951, **63**: 577–585.
57. Placido Sousa, C., and Evans, D. G.: The action of diphtheria toxin on tissue cultures and its neutralization by antitoxin. Brit. J. Exptl. Path., 1957, **38**:644–649.
58. Pope, C. G., and Stevens, M. F.: The purification of diphtheria toxin and the isolation of a crystalline toxin-protein. Brit. J. Exptl. Path., 1958, **39**:139–149.
59. Pope, C. G., and Stevens, M. F.: The properties of crystalline diphtheria toxin-protein: Its antigen composition as determined by gel-diffusion methods. Brit. J. Exptl. Path., 1958, **39**: 150–157.
60. Pope, C. G., and Stevens, M. F.: The properties of crystalline diphtheria toxin-protein: Observations on the phosphate-stable antigen. Brit. J. Exptl. Path., 1958, **39**:386–392.
61. Pope, C. G., and Stevens, M. F.: The properties of crystalline diphtheria toxin-protein: Observations on the pepsin and trypsin-stable antigens. Brit. J. Exptl. Path., 1958, **39**:490–496.
62. Pope, C. G., and Stevens, M. F.: The properties of crystalline diphtheria toxin-protein: The reaction of the phosphate and of pepsin-stable antigens with diphtheria antitoxic serum. Brit. J. Exptl. Path., 1959, **40**:410–416.
63. Relyveld, E. H.: Toxine et antitoxine diphtériques. Étude immunologique. Hermann, Paris, 1959.
64. Report: World incidence of diphtheria during recent years. Epidemiol. Vital Stat. Rept., W. H. O., 1952, **5**:223–236.
65. Russell, W. T.: The epidemiology of diphtheria during the last forty years. Med. Res. Council (Great Britain) Spec. Rept. Ser. No. 247. 1943.

66. Sayers, E. G.: Diphtheritic myocarditis with permanent heart damage. Ann. Internal Med., 1958, **48:**146–157.
67. Seligmann, E.: Types of diphtheria bacilli in New York in 1940. Amer. J. Hyg. Sec. B, 1941, **34:**125–137.
68. Strauss, N., and Hendee, E. D.: The effect of diphtheria toxin on the metabolism of HeLa cells. J. Exptl. Med., 1959, **109:**145–163.
69. Tasman, A., and Brandwijk, A. C.: Experiments on metabolism with *Corynebacterium diphtheriae*. J. Infect. Dis., 1940, **67:**282–291.
70. Tasman, A., and Lansberg, H. P.: Problems concerning the prophylaxis, pathogenesis and therapy of diphtheria. Bull. W. H. O., 1957, **16:**939–973.
71. Taylor, E. M., and Moloney, P. J.: An investigation of the League of Nations standard requirements for Schick toxin. J. Immunol., 1937, **33:**191–202.
72. Tuuri, A. L., Johnston, H. L., and Hartung, D.: Adapting immunization programs to special groups. Pub. Health Repts., 1957, **72:**283–289.
73. Wildführ, G.: Zür Frage der Bakteriämie im Beginn der Diphtherie. Zent. f. Bakt. I Abt. Orig., 1949, **154:**14–17.
74. Wood, N., and O'Gorman, G.: Erythromycin treatment of a diphtheria carrier epidemic in a mental deficiency hospital. Antibiotic Med., 1957, **4:**465–469.

Chapter 31

MYCOBACTERIUM

THIS GENUS includes a number of species of related bacteria which are most conveniently considered in three groups. The first includes the mammalian tubercle bacilli *Mycobacterium tuberculosis* var. *hominis* and *Myco. tuberculosis* var. *bovis,* and the avian tubercle bacillus, *Myco. avium.* In the second group there are Hansen's bacillus or *Myco. leprae* and the rat leprosy bacillus, *Myco. leprae murium.* The third group is made up of Johne's bacillus, or *Myco. paratuberculosis,* and certain acid-fast bacilli isolated from cold-blooded animals, together with the saprophytic acid-fast forms.[14]

THE TUBERCLE BACILLI

Tuberculosis is an old disease of man and is still one of the most widespread; it is estimated that over a million persons in the United States have active tuberculosis, and about half of these are known to the health departments. Its infectious nature was suspected by Fracastorius in the early part of the sixteenth century, and Villemin showed, in 1865, that the disease could be transmitted by the inoculation of tuberculous material. It was in 1882 that Koch demonstrated the tubercle bacillus by special staining methods, isolated and grew it in pure culture, and reproduced the disease by the inoculation of the bacilli.

Morphology and Staining.[7, 26] The tubercle bacilli are slender, sometimes slightly curved, rods 2 to 4 μ in length and 0.3 to 1.5 μ in breadth. They occur singly but are often found in small groups, sometimes in compact masses in which the individual bacilli cannot be distinguished. The bacilli of the human variety tend to be somewhat longer and more slender than those of the bovine type, but the morphology of both is variable, and no distinction can be made on this basis. The bacillary form is generally retained in the tissues; in culture longer filamentous forms are sometimes seen together with swollen or club-shaped cells resembling the diphtheria bacillus. Branched forms are present in cultures of the avian tubercle bacillus but are rarely seen in cultures of the mammalian bacilli. The occurrence of filamentous forms and true branching indicates the close relation of these bacilli to the higher fungi.

The tubercle bacillus is nonmotile and non-spore-forming and produces a capsular substance in artificial cultures, particularly when grown upon serum mediums. The granular structure of the individual cells is marked. Vacuoles often occur in abundance and may even give the stained cell the appearance of a chain of cocci. The significance of the small, deeply staining bodies sometimes observed within the cells is not clear; they do not show the enhanced resistance characteristic of spores.

The tubercle bacilli cannot be stained by the usual staining methods that are

effective with other bacteria, for there is a marked resistance to the penetration of dyes into the cell that is associated with the presence of relatively large amounts of unsaponifiable wax. The cells may be stained in 2 or 3 minutes by steaming carbol-fuchsin or by 18 hours' exposure to the dye at room temperature. Once stained, the bacilli are difficult to decolorize and resist the action of alcohol and dilute solutions of mineral acids and for that reason are termed "acid-fast." They may be demonstrated in smears by the Ziehl-Neelsen method, in which the smear is stained with hot carbol-fuchsin, decolorized with acid alcohol, and counterstained with a dye of contrasting color. Methylene blue is most commonly used but some workers prefer other stains such as picric acid and Bismarck brown. The bacilli may also be stained with carbol-auramine, a dye that fluoresces a brilliant yellow in weak ultraviolet light. This method is seldom used, and then only for screening purposes.[67] Non-acid-fast bacilli may be observed in young cultures.

Non-acid-fast but gram-positive granules, known as Much granules, were described by Much in 1907 as occurring in the material from cold abscesses and elsewhere in which acid-fast bacilli could not be demonstrated but which, nevertheless, proved to be infective. Considerable numbers of acid-fast bacilli, perhaps 100,000 per ml., must be present, however, before there is a reasonable chance of finding them in smears. Much maintained that these granules are viable and virulent and give rise to typical acid-fast rods. They have been observed by others, but their significance is open to question. Some regard them as degeneration products or artifacts of the staining procedure.

In broth cultures there is a thick, wrinkled skin of surface growth which tends to spread up the sides of the flask; masses of bacilli may become detached and sink to the bottom as a lumpy sediment. Growth on the surface of solid mediums is generally dry and granular with nodular, heaped-up areas. The human variety of the tubercle bacillus usually produces a pale yellow or orange-yellow growth on serum-containing mediums and a creamy or white growth in the absence of serum. The bovine variety is not pigmented on serum mediums. Some avian strains give a faint pink-colored growth on egg mediums. A peculiar almond-like odor is often noticeable in cultures of these bacteria.

Physiology. The tubercle bacillus is an aerobe and will not grow under completely anaerobic conditions. The mammalian varieties grow best at 37° C. and not at all below 30° C. or above 42° C.; the optimum temperature for the avian type, however, is 40° C. Growth is relatively slow, and 4 to 6 weeks are generally required for an abundant growth, although minute colonies appear in 8 to 10 days. Most strains of the avian type

Figure 177. *Mycobacterium tuberculosis.* Acid-fast stained smear of tuberculous sputum. × 1050.

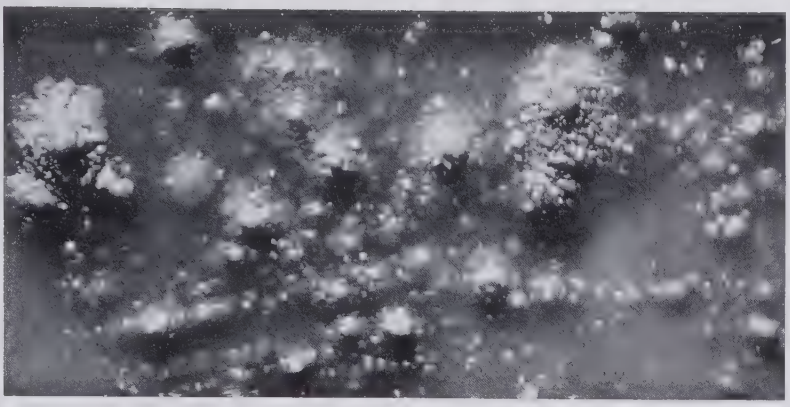


Figure 178. Colonies of the human variety of the tubercle bacillus, H-37 strain, on Löwenstein's medium, 5 weeks' incubation. × 3.

adapt themselves readily to culture on artificial mediums and in time are able to grow much more rapidly, but others remain slow-growing.

Enriched mediums are required for primary isolation, and these usually contain eggs, glycerol, and sometimes dyes to inhibit the growth of contaminants. The commonly used mediums are the Jensen modifications of Löwenstein's medium, containing egg, potato meal, bone marrow infusion, citrate, glycerol and asparagin and malachite green; the egg yolk–potato medium of the American Trudeau Society; and McNabb's or Frobisher's modification of Petragnani's medium. Glycerolated potato is used by the French workers. Corper's medium is glycerolated potato with the modification that the pieces of potato are soaked for a short time in a solution of crystal violet before sterilization with the glycerol solution.

The human variety of the tubercle bacillus grows more abundantly on these mediums than does the bovine variety, and for that reason it is termed "eugonic" and the bovine type "dysgonic." These two varieties also differ in that glycerol is markedly favorable to the growth of the human type but does not so affect the bovine type.[108] It is not known why glycerol exerts this favorable effect; attempts to substitute related compounds such as isopropyl alcohol, propyl alcohol, glycol, trimethylene glycol, and inositol have not been successful. Glucose, however, acts in much the same manner as glycerol. The metabolism of a variety of carbon compounds, including those functional in the Krebs cycle, has been studied in detail by Youmans and Youmans.[138, 139, 141] Egg yolk has been reported to contain a lipid growth factor, but this appears to stimulate rather than be essential to growth. Thiamin, pyridoxin, and riboflavin do not stimulate growth.

Growth occurs much more readily and upon simpler mediums after primary isolation. The human tubercle bacillus grows well upon nutrient agar or broth containing glycerol (2 to 5 per cent) and has been cultivated in a variety of synthetic solutions. One of the best known of these is Long's synthetic medium which contains glycerol, asparagin, citrate, and inorganic salts. Dubos and his co-workers[37, 39, 40] have found that growth is facilitated and occurs diffusely throughout the liquid medium in the presence of certain water-soluble lipids. A medium containing asparagin, glucose, phosphate, citrate, bovine serum albumin, magnesium sulfate, and a lipid commercially designated Tween 80 (a polyoxyethylene derivative of sorbitan mono-oleate), though it has appreciable growth-inhibiting activity, will give visible growth of the human variety of tubercle bacillus within 2 weeks. The medium becomes inhibitory in time owing to the activity of lipase contaminating bovine albumin preparations, which liberates oleic acid to bacteriostatic concentrations from the Tween 80;

the effect can be eliminated by the use of commercial crystalline bovine serum albumin, the addition of 0.01 per cent sodium fluoride and by other means. The bovine type grows poorly or not at all on these mediums and is benefitted only slightly by the presence of glycerol. The avian type of tubercle bacillus, however, grows better than the human type after a few transfers, and good growth may be obtained on nutrient agar in the absence of glycerol. Like the human type the avian bacillus grows much more profusely in the presence of glycerol. These cultural differences in the three types of tubercle bacilli are of little significance in their early differentiation, for several transfers over a period of months are necessary before they are obvious.

The biochemical reactions of the tubercle bacilli have not been studied at length. Growth occurs in milk, but no visible change is produced. Indol is said not to be formed. In glycerol broth, cultures of the bovine type become alkaline while those of the human type become slightly acid. The conventional fermentation test is not readily applicable to the tubercle bacilli because of their very slow growth. The necessary prolonged incubation tends to give inconsistent results, possibly because of further decomposition of organic acids, concurrent deamination, etc. The use of very heavy, nongrowing suspensions of the bacilli in carbohydrate-containing mediums has indicated the possibility of useful differentiation. It is reported[121] that strains pathogenic for man but not for chickens ferment trehalose but not xylose, while strains pathogenic for chickens but not for man ferment xylose but not trehalose.

Growth in cell culture. The growth of tubercle and related bacilli in animal tissue cells in culture has been of interest since the early studies of Maximov on the pathogenesis of such infections. More recently cultures of spleen and liver explants,[71] monocytes,[10] and cells of the HeLa line[109, 115, 116] have been used as substrates with particular reference to the differentiation of strains with respect to virulence. Infection of the host cells in maintenance mediums occurs within the first day or so, and proceeds rapidly in growth mediums. It has been observed consistently that virulent strains grow more rapidly, with appreciable growth in three to five days, and there is concurrent destruction of the host cells. The tissue culture method appears to be less sensitive than conventional culture for primary isolation.

Chemical Composition. The chemical composition of the tubercle bacilli has been more intensively investigated than that of any other bacteria. These bacilli are of particular interest in this connection because of their high content of lipoidal substances which may make up as much as 40 per cent of the dry weight. Protein, a considerable proportion of which is nucleoprotein, makes up about half the dry weight and polysaccharides are found in relatively small amounts.[112]

The lipids have been studied at length by Anderson[2, 3] and his co-workers. In addition to neutral fat, two general types of material may be distinguished:

(1) Phospholipid, containing saturated and unsaturated fatty acids including the well known palmitic, linoleic, and linolenic acids, together with two acids peculiar to the tubercle bacillus, *phthoic acid,* isomeric with cerotic acid, and *tuberculostearic acid,* isomeric with stearic acid and optically inactive.

(2) An acid-fast wax, containing polysaccharides hydrolyzing to mannose, arabinose, and galactose; a soft wax which is a complex glyceride; and an unsaponifiable wax (acid-fast) made up of higher alcohols and including a high molecular weight saturated hydroxymethoxy acid termed *mycolic acid,* a higher alcohol designated *phthiocerol,* and a levorotatory fatty acid, *mycocerosic acid.* Acid-fastness is associated with the presence of mycolic acid.

Some of these substances appear to be physiologically active. The unsaponifiable acid-fast wax apparently stimulates the multiplication of undifferentiated connective tissue cells, and phthoic acid induces a proliferation of epithelioid cells.[106, 107] A toxic lipid fraction, extractable in monochlorbenzene, is

thought to contribute to the primary toxicity of massive inoculums, but is of uncertain significance in the disease.[120] A yellow pigment is found in the neutral fat which is designated *phthiocol* and is a hydroxynaphthaquinone which may be reversibly reduced at a relatively low potential. This substance is identical with vitamin K except for the substitution of a hydroxyl group for the phytyl radical on the third carbon.

Polysaccharide mixtures, containing immunologically active and inactive substances, have been isolated from mammalian tubercle bacilli, but their significance is not as yet understood. The protein constituents of the cell appear to be the most important immunologically and have been studied in connection with the preparation and activity of the various tuberculins.

Cord factor.[15, 38, 126] The tendency of tubercle bacilli to grow in filaments, or cords, in liquid culture was found by Middlebrook, Dubos, and Pierce in 1947 to be associated with the virulence of human and bovine varieties of the microorganism. On treatment with petroleum ether, the filaments break up, and a lipid substance is extracted which is acid-fast and toxic to leucocytes and mice. This substance was designated the cord factor by Bloch. Its association with virulence is not altogether clear for it may be extracted from the attenuated BCG strain (see below) and from nonpathogenic forms such as the smegma bacillus.

Resistance. Although having much the same degree of resistance to heat as the vegetative cells of other bacteria, the tubercle bacilli are relatively highly resistant to drying, chemical disinfectants, and other deleterious environmental influences, very likely as a consequence of their contents of wax. In putrefying sputum the bacilli may remain viable for weeks or months and in dried sputum kept in a cool dark place for as long as 6 to 8 months. Sputum that is completely dried, so that particles are capable of floating as dust in the air, may be infective for 8 to 10 days. In dried sputum they may survive 100° C. for an hour but are killed in the usual way by moist heat. Phenol penetrates the bacilli only slowly, and a 5 per cent solution requires 24 hours to kill the bacilli in sputum. The action of other disinfectants is similarly retarded, and hypochlorites and certain synthetic detergents have almost no effect on these bacteria. Tubercle bacilli are readily killed by exposure to direct sunlight; bacilli from cultures are killed within 2 hours but in sputum may survive 20 to 30 hours of such direct exposure.

A number of different kinds of substances are bacteriostatic for the tubercle bacilli, including streptomycin, *p*-aminosalicylic acid (PAS), sulfones, thiosemicarbazones, and certain pyrimidine derivatives such as isoniazid. These substances are extremely useful for chemotherapeutic purposes (see below).

Variation. The variability of the tubercle bacilli has been studied extensively, but with inconclusive results. Colonial variants, thought by some to be analogous to the S and R variants of other bacteria, have been observed, and it has been claimed by some workers that the S type is the more virulent, and by others that virulence and colonial morphology are independent. The colonial morphology of the tubercle bacilli is, to a considerable degree, a transient adaptation to environmental conditions, and prompt alteration of colonial appearance results on transfer to a different medium. For example, it has been observed that colonies growing in the presence of ether extract of egg yolk are smooth and markedly different from the usual colonial type, but the effect is only a temporary physical one. The status of the S-R variation in the tubercle bacilli is, then, by no means clear as yet.

Bacille Calmette-Guérin. A bovine strain of the tubercle bacillus was rendered completely avirulent by Calmette, who cultivated it over a long period (230 transfers in 13 years) on bile-glycerol-potato medium. This strain is designated as BCG (*Bacille Calmette-Guérin*) and has been of particular interest in connection with active immunization against tuberculosis. The nature of the change

which resulted in the loss of virulence is completely unknown. The loss appears to be permanent and virulence does not reappear on transfer to ordinary mediums.

Life cycles. The pleomorphic tendencies of the tubercle bacilli, coupled with the occurrence of non-acid-fast rods in young cultures and the granular elements described by Much, have been interpreted by a number of workers as indicative of a cyclic succession of morphological types, or life cycle, through which these bacilli go. As pointed out elsewhere (Chap. 7), evidence suggesting the occurrence of life cycles of bacteria is subject to various interpretations.

Filterable forms. L variation[76] occurs during which viable granules, perhaps to be regarded as Much granules, are produced. The viable fragments, including filaments as well as granules, revert to the bacillary form on culture on the conventional mediums. The latter probably account for the many reports of filterable forms, or "ultravirus" of tubercle bacilli.

Drug resistance. Like other bacteria, the tubercle bacilli may become resistant to chemotherapeutic drugs *in vitro* and *in vivo*.[48, 92] The latter is more common with these bacteria than with other kinds because of the nature of the disease and the prolonged treatment required, and because resistance to certain of the effective drugs, streptomycin and isoniazid in particular, is especially prone to occur. Isoniazid resistance is accompanied by a loss of catalase activity by tubercle bacilli, but some catalase activity persists in resistant saprophytic mycobacteria. There is evidence that the latter contain two catalase systems, one of which is not isoniazid-sensitive.[124]

The practical importance of drug resistance depends upon the drug and its toxicity. For example, a blood level of 10 to 15 μg. per ml. of streptomycin may be attained, and sensitive strains are inhibited *in vitro* by 0.5 μg. per ml., slightly resistant strains by 2 to 4 μg. per ml., moderately resistant by 200 to 400 μg. per ml., and more than 50,000 μg. per ml. are required to inhibit the growth of highly resistant strains. Blood levels of isoniazid of 3 μg. per ml. may be attained, and sensitive strains are inhibited by 0.025 μg. per ml. *in vitro*. Thus a proportionately higher resistance must occur in the case of isoniazid than of streptomycin to allow growth in therapeutic concentrations. The assay of drug sensitivity is complicated by the slow growth of the bacilli, and more rapid methods have been devised based on the use of suspensions of bacilli and indicators of oxidative metabolism such as resazurin or tetrazolium.[63, 73, 127]

The facility with which resistance develops to a single chemotherapeutic agent is such that for practical purposes it is necessary to reduce the development of drug resistance by the use of combinations of drugs. As described elsewhere (Chap. 7), if the resistant strain arises as a consequence of the selection of a chance mutant by the drug-containing environment, and resistance to one drug is independent of that to another, the probability of occurrence of a double mutant is given by the product of the two mutation rates and becomes extremely small. The development of drug resistance *in vivo* is, in fact, markedly inhibited by the use of two drugs in combination, commonly streptomycin plus PAS, isoniazid plus PAS, etc. Resistant strains do not appear to differ significantly from the sensitive parent strain except in the case of those resistant to isoniazid which are appreciably less virulent, though still capable of producing fatal disease in man.[83, 123]

Pathogenicity for Man. In the United States tuberculosis is one of the leading causes of death and is one of the first three in a relatively large portion of the life span, ages 15 to 49. It is first in the 15 to 24 age group, second in the 35 to 39 group, and third in the 40 to 49 group. It is estimated that fifty-five million persons in this country have, or have had, tuberculosis, that there are 1,200,000 persons with active or inactive infection requiring treatment or supervision, and about 800,000 active cases of infectious

tuberculosis. Of the last, possibly 250,000 are not under treatment and actively spread the disease.

The mammalian tubercle bacilli, both bovine and human varieties, are pathogenic for man. The human type is practically always responsible for pulmonary tuberculosis in adults and is usually found in children also. The bovine variety may occur occasionally in pulmonary tuberculosis in children but is more often found in infections of other tissues. Mixed infections with the two types of tubercle bacilli have been reported but are rare.

The avian tubercle bacillus is generally considered to be, for all practical purposes, nonpathogenic for man. Cases of human infection, however, are reported from time to time,[34] and some studies have suggested that it may be more important in human disease than generally believed. In one study carried out in Germany, 8.5 per cent of 218 positive specimens were avian tubercle bacilli.[87]

Routes of infection. The tubercle bacillus may enter the body by way of the genitourinary tract, the conjunctiva, the skin, the alimentary tract, and the respiratory tract. Primary infection of the genitourinary tract is possible but, under natural conditions, rarely occurs. Infection through the conjuctiva takes place readily under experimental conditions; its frequency under natural conditions is not known, for the cervical lymph glands, where the infection would first appear, are readily infected by other channels. Infection through the skin is relatively rare; whether the bacilli can penetrate the intact skin is uncertain, but they may enter through abrasions or other traumatic injuries. Primary infection of the skin generally results in verruca tuberculosa (pathologist's wart) or lupus vulgaris.

Primary infection by the alimentary tract is a consequence of the ingestion of tubercle bacilli in infected food, most commonly milk, and occurs with considerable frequency in children. Secondary infection may occur, especially in children, by swallowing tuberculous material of respiratory origin. In the upper portion of the alimentary tract the bacilli enter the body tissues through the faucial, pharyngeal, and lingual lymphoid follicles and first affect the upper cervical and retropharyngeal lymph glands. Tuberculous lesions of the tonsils are not uncommon though rarely conspicuous. The stomach is rarely a portal of entry, but the bacilli penetrate the intestinal mucosa by way of Peyer's patches.

The respiratory tract is the most frequent and most important route of infection with the tubercle bacillus and the facility with which this occurs is demonstrable under both controlled[101] and natural[102] conditions. The coarser infectious particles in inspired air are filtered out and deposited on the nasal, buccal, and pharyngeal surfaces and the

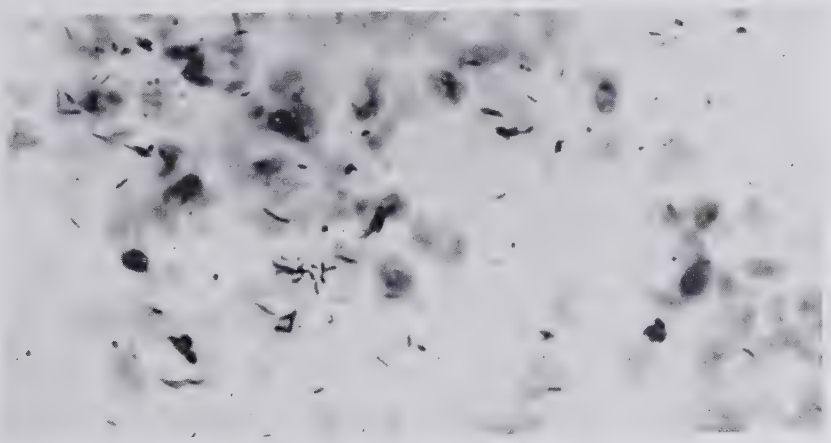


Figure 179. Tubercle bacillus. Acid-fast stained smear of pus from a liver abscess in a rhesus monkey. × 1050.

bacilli, upon penetration, set up focal infections in the local lymphatic tissue. Fine droplets or particles of dust, however, may, and frequently do, enter the lungs directly (Chap. 10).

The spread of infection in the body. Tubercle bacilli are disseminated through the body from primary or secondary foci of infection by way of the lymph, the blood stream, or directly by extension along contiguous surfaces. Distribution via the lymphatics occurs more readily in children than in adults, and the bacilli may localize at almost any point but most commonly in the lymph nodes. Bacilli present in the thoracic duct may gain entrance to the blood stream. The blood stream may also be invaded directly through erosion of a vessel wall by a focus of infection. The bacilli are transported throughout the body by the blood and give rise to acute miliary or chronic disseminated tuberculosis. An example of the pathogenesis of hematogenous spread occurred following an instance of intravenous self-inoculation with suicidal intent;[58] general miliary infiltration throughout both lungs occurred between the sixteenth and twenty-first days, but the tuberculin reaction did not become positive until the twenty-sixth day.

Spread by extension occurs most frequently in ulcerative pulmonary tuberculosis with the breaking down of foci of infection and the consequent presence of bacilli in the sputum. The pleura and pericardium may be invaded directly from a focus of infection in the lungs. The infection of such serous surfaces may be localized and fibrinous and result in adhesions or may assume an acute miliary form. Direct extension may also occur elsewhere, as from the kidneys to the ureters and bladder or into the peritoneal cavity and adjacent areas from intestinal ulcers. Practically every organ and tissue of the body may be invaded by the tubercle bacillus.

Tuberculosis in man is most commonly the pulmonary form with a primary invasion of the apices, and over 90 per cent of the deaths from tuberculosis are due to the pulmonary type. Whether primary infection is a direct consequence of inhalation as believed by Koch, or whether it is in most cases of hematogenous origin following a preliminary infection of the lymphatic system as urged by Calmette, is not altogether clear, but on the whole the evidence favors inhalation infection. Although pulmonary tuberculosis is by far the most common form of infection in adults, it is somewhat less frequent in children, but precisely how much less is not known with certainty. Pulmonary infection in children differs from that in adults in that the hilum glands are involved. Involvement of the lymphatics is frequent in children. The anatomical distribution of the lesions usually takes one or the other of two well-defined forms; in the one the lesions are found predominantly in the tracheobronchial lymphatics and in the other in the mesenteric lymph nodes. In general, children show a tendency toward generalized infection.

Other tissues and organs are involved less frequently. The spleen, liver, and kidneys are sometimes infected. Tuberculosis of the adrenals gives rise to Addison's disease. Infection of the skin or lupus is not uncommon. Bone and joint tuberculosis is more common in children than in adults, and tuberculosis meningitis is not infrequent in the young. It will be clear that tuberculous infection may take a variety of clinical forms.

The tubercle. Lesions caused by the tubercle bacillus, in whatever part of the body they occur, usually possess a definite although not absolutely characteristic appearance and histological structure. Small nodules or tubercles, plainly visible to the naked eye, are so uniformly observed in advanced infections with the tubercle bacillus that their presence has given the name to the disease. The young tubercle probably originates from the fixed cells surrounding the invading bacilli. By the proliferation of the fixed cells, elongated "epithelioid" cells are developed in more or less definite concentric layers and come to form the substance of the tubercle. So-called giant cells or foreign-body giant cells soon appear in the developing tubercle; these

are huge multinuclear masses of protoplasm thought by some to be especially distinctive of true tubercle formation, though it is doutbful if this criterion can be maintained. Either they are produced by the fusion of a number of macrophages or they are of single-cell origin. While the formation of epithelioid and giant cells is going on, leucocytes, at first polymorphonuclear leucocytes and later lymphocytes, cluster around the periphery of the tubercle. Degeneration of the tubercle eventually sets in, the central portion becomes necrotic, and this is followed by caseation and then by softening of the caseous mass.

In some cases calcium salts are deposited in the tubercle (calcification), converting it into a hard, dry, friable body which may become encapsulated and completely walled off from the surrounding tissues. In other instances, however, this healing process does not take place, and there is instead an extension with coalescence and the formation of confluent masses that may reach a diameter of 4 or 5 cm. The erosion of a blood vessel may occur, and the discharge of large numbers of the bacilli into the blood stream leads to a general diffusion of small tubercles of the size of millet seeds (acute miliary tuberculosis).

The early stages of tubercle formation, characterized by cell proliferation and leucocytic infiltration, are probably a response to a chemical or mechanical stimulus caused by the presence of the bacilli; the later changes, leading to necrosis and caseation, may be attributed to the action of the bacterial products. The host becomes sensitized to the bacillary substance, and the allergic response of the tissues is involved to no small extent.

Predisposing factors. Few diseases are as completely dominated by predisposing factors as tuberculosis. Infection with the tubercle bacillus is exceedingly common, and there are few adults, particularly those living in cities, who escape. The proportion of individuals coming to autopsy who show evidence of infection is very high and has been reported as high as 97 per cent. In one series of cases, for example, it was found that 25 per cent of 10-year-olds, 55 per cent of 20-year-olds, 80 per cent of 30-year-olds, and over 90 per cent of individuals over 50 years of age show calcified lesions of infection. The bacilli, then, are present in the great majority of adults and also in many children, but the development of clinical tuberculosis is restrained by the nonspecific resistance of the host. Predisposing factors, therefore, are those which tend to interfere with normal physiological well-being and include such things as insufficient or unsuitable food, prolonged exposure to dampness, a sedentary life, and chronic fatigue. In the First World War the incidence of tuberculosis rose in this and other countries, and in the Second World War the same tendency was again apparent in Europe. In both man and animals tuberculosis is a disease associated with confinement, of men in houses and cattle in stables. The opportunities for transmission of the infection are, of course, much greater in community life.

There is a marked occupational predisposition to tuberculosis in the dusty trades, and the constant inhalation of almost any kind of dust results in an increased incidence of pulmonary tuberculosis. Silica dusts, however, appear almost specifically to predispose to tuberculosis, and the incidence of infection in those constantly exposed to such dusts is much higher than that in the general population.

It has long been suspected that there are familial or hereditary tendencies to tuberculosis in man. Resistance to infection in experimental animals is to some degree genetically determined, but the conclusive demonstration of a similar phenomenon in man is difficult owing to both a long generation time and the inability to carry out appropriate breeding experiments. The incidence of new infection in tuberculous families in considerably greater than in the general population, but whether this is a consequence of increased risk alone or in part of genetically determined factors is not clear. In any case the disease itself is not inherited, and congenital tuberculosis occurs only rarely.[132]

The relation of childhood infection to clinical tuberculosis in the adult has been a matter of considerable interest, and it has been suggested that pulmonary tuberculosis of the young adult may, in many instances, be a consequence of the lighting up of an old infection. The evidence, however, is against this view, and it is probable that tuberculosis in the adult is a consequence of reinfection rather than of a flaring up of the healed or partially healed lesions from childhood infection. A considerable period, possibly years in some instances, may elapse between reinfection and the appearance of tuberculosis in clinical form.

Bacteriological Diagnosis.[82, 96, 100] Tubercle bacilli are discharged from the infected individual in sputum and urine and may be demonstrated in these materials and in gastric washings, spinal fluid, or infected tissues, depending on the location of the infection. Of specimens from these sources, those obtained by gastric lavage are of particular value in respiratory tuberculosis in children, who tend to swallow sputum, and are also useful in adult infections. Results must be interpreted with caution because of the presence of saprophytic acid-fast bacilli in the gastrointestinal tract derived from the ingestion of foods, especially fruit, on which these forms occur; furthermore, there is some reason to believe that some fats and oils may impart acid-fast properties to ordinarily non-acid-fast bacteria present in the alimentary tract. The presence of the bacilli may be shown either directly or after concentration by examination of stained smears, culture, and guinea pig inoculation.

As a rule, some method of concentration is desirable,[23] for not less than 100,000 bacilli per ml. must be present before there is a reasonable chance of finding them by microscopic examination. The resistance of the tubercle bacillus allows treatment of the specimen with hypochlorite or NaOH to destroy contaminants and digest the viscous sputum to facilitate concentration by centrifugation. The sediment is used to prepare smears for direct examination, and to inoculate culture mediums and/or guinea pigs.

Smears are usually stained by the Ziehl-Neelsen method. The demonstration of acid-fast bacilli allows a provisional diagnosis but does not indicate whether the bacilli were viable or whether they are virulent tubercle bacilli. Their presence in urine specimens in particular must be interpreted with caution because of the frequent occurrence of the smegma bacillus which is also acid-fast.

Guinea pigs are inoculated in the groin or in the muscle of the thigh. If a reasonable number of tubercle bacilli have been inoculated, enlargement of the regional lymphatics may be noted in 2 or 3 weeks, the pig becomes emaciated by 4 to 6 weeks and usually dies not long after. With very small numbers of bacilli evidence of the infection may be delayed 2 or 3 weeks longer. If it is not obviously ill or does not die, the animal should be kept for 8 weeks before sacrificing. At autopsy the regional lymph nodes will be found to be enlarged and filled with caseous material. Necrotic areas in the spleen and liver are characteristic of the gross pathology in this animal; tubercles are seldom seen, and the lungs are only slightly affected and the kidneys almost never. Tubercle bacilli may be cultured from the lesions and found in acid-fast-stained smears. Some workers tuberculin-test the animals before inoculation and 3 to 4 weeks afterward, the development of hypersensitivity indicating infection. If differentiation of the human and bovine varieties of the bacillus is desired, rabbits may be inoculated.

A variety of mediums, containing glycerol and egg, may be used for culture, the particular medium varying from one laboratory to another. The culture is commonly enclosed, as in a screw-cap culture tube or with paraffin poured over the cotton plug, to minimize drying, but a small hole needs to be left to allow air exchange. Characteristic colonies of tubercle bacilli appear after about 3 weeks' incubation. Extended incubation, to as long as five months,

may give a few additional positive cultures.[97] Culture is not identification, of course, and acid-fast bacilli, which grow like the tubercle bacilli, are occasionally found in nontuberculous lesions.

Chemotherapy.[77, 133] Effective chemotherapy of tuberculosis requires that the chemotherapeutic agent be diffusible into the tuberculous process as well as having specific antibacterial activity. In addition, the necessity for administration over relatively long periods accentuates the problems of toxicity and the development of drug resistance by the microorganism. Prior to 1938 no substances of practically significant activity were known, but while the ideal chemotherapeutic agent for this disease has not been found, a number of drugs of reasonable efficacy are now available. These fall into two groups, *viz.*, the synthetic compounds and the antibiotics.

The synthetic tuberculostats. There are four groups of these: the sulfones, the aminohydroxybenzoic acids, the thiosemicarbazones, and the pyrimidine carboxylic acid derivatives. The observation that sulfanilamide affected the progress of experimental tuberculosis led to the preparation and trial of related compounds. Of these, the sulfones, 4, 4′-diaminodiphenyl sulfone and its derivatives such as Promin, Diasone, and Sulphetrone, were found to be partially effective chemotherapeutic agents. All are toxic, the most common effect being on the erythrocytes, and, while toxicity may be reduced by adjusting dosage, it has not been possible to eliminate it, probably because the activity of derivatives is a consequence of their degradation *in vivo* to the parent compound.

The observation that benzoates and salicylates stimulate the respiration of the tubercle bacillus *in vitro* provided the basis for the discovery of the chemotherapeutic activity of *p*-aminosalicylic acid, the most active of the compounds tested. This substance is of low toxicity and is readily absorbed from the gastrointestinal tract. It has appreciable chemotherapeutic activity when given alone, but its greatest utility is in combination with streptomycin or isoniazid.

The thiosemicarbazones are more effective chemotherapeutic agents than the sulfones or salicylates and have been used relatively widely in Europe but not in this country. The compound *p*-acetaminobenzaldehyde thiosemicarbazone (Tibione) has been tested extensively together with a number of derivatives. All of these are relatively toxic substances and give severe side reactions including anemia and liver and kidney damage in addition to gastrointestinal disturbances.

A number of pyrimidine derivatives, isonicotinic acid hydrazide (isoniazid) and related compounds such as the isopropyl derivatives, are available under a number of proprietary names and are widely used and effective chemotherapeutic agents although they have some neurotoxicity.

Antibiotics. Streptomycin was the first of the antibiotics to be used for the therapy of tuberculosis, and continues to be the most widely employed. Most other antibiotics effective in the treatment of acute infectious diseases are not satisfactory, but the broad spectrum antibiotic, cycloserine (D-4-amino-3-isoazolidine), has been used to a considerable extent as an effective chemotherapeutic agent.

As noted earlier, the various chemotherapeutic agents are commonly used in combination in the treatment of tuberculosis to minimize the occurrence of drug-resistant variants.[22, 72] While commonly given separately, very many compounds or complexes of two drugs have been prepared under a variety of names, and are described elsewhere (Chap. 6).

Antibacterial activity in vivo. Assay of the chemotherapeutic efficiency of tuberculostatic drugs is dependent upon the nature and stage of the infection. For example, in early infection, growth of the bacilli is largely intracellular and streptomycin and PAS do not penetrate macrophages, though isoniazid does, to affect the phagocystosed bacilli, but when the individual is allergic (see below), growth tends to be more frequently extracellular. Similarly, in tuberculous lesions the bacilli grow most

densely at the periphery of the caseous zone, and as the lesion expands they are engulfed in caseous material and lie dormant. Such dormant bacilli are not appreciably affected by the bacteriostatic activity of drugs, and the concentration of drug is lower in such material than in the blood. Thus a focus of infection may persist in the individual undergoing chemotherapy and result in relapse, and possibly infection with drug-resistant bacilli, when therapy is discontinued. The intracellular persistence of viable bacilli during chemotherapy may be of some small significance in pulmonary tuberculosis, but of very considerable importance in tuberculosis meningitis.

The anatomical changes occurring in the tuberculous lesion during chemotherapy have been summarized by Auerbach[5] who groups them as follows: (1) a rapid and extensive clearing of the perifocal reaction; (2) a greatly decreased width of fibrous capsules around necrotic foci; (3) decreased thickness of the cavity wall and overlying pleura; (4) a decrease in pulmonary fibrosis and emphysema; (5) a marked decrease in the occurrence of massive pulmonary hemorrhages; (6) a difference in the mode of cavity healing; and (7) an accelerated healing of the tuberculous process. The last is especially evident when treatment is begun early in the disease and appears as an accelerated development of collagen fibrils and a corresponding decrease of cells and capillaries of the granulation tissue around the necrotic foci and the walls of tuberculous cavities. The changes differ somewhat depending upon the chemotherapeutic agent.[69]

Immunity.[114, 130] The immune response of the animal body to the presence of the tubercle bacillus is indicated by the appearance of agglutinins, precipitins, opsonins, and complement-fixing antibodies in the serum. This response is not marked, however, for these antibodies are present only to low titers. Erythrocytes sensitized with tuberculin are passively agglutinated,[81] but this reaction appears to have only limited diagnostic or prognostic value.

The most striking immune response is the development of hypersensitivity of the delayed type to the bacillary cell substance. Within limits, this allergic response is protective against reinfection, as shown by Koch's early experiments. He showed that the subcutaneous inoculation of the normal guinea pig with tubercle bacilli produces no immediate response, but that in 10 to 14 days a nodule develops which breaks down to a persistent tuberculous ulcer, and the regional lymph glands become swollen and caseous. In the tuberculous animal, however, an indurated area appears within a day or two, and there is slight necrosis with the formation of a shallow ulcer which heals promptly without the development of gross tubercle tissue or the invasion of the adjacent lymphatics by the bacilli. This is known as the Koch phenomenon.

This resistance to reinfection is relative and is not shown unless the primary infection is of some weeks' standing or large numbers of bacilli are injected. Animals may be sensitized, not only by infection with virulent bacilli, but also by the inoculation of attenuated or killed bacilli. Sensitization with preparations of the bacillary cell substance is difficult and very large doses must be administered. Raffel[93] has shown that the reaction is a result of inoculation with tubercle bacillus protein combined with a purified wax fraction consisting of an ester of polysaccharide and higher alcohols with hydroxy fatty acids; the protein alone produces an immune response with formation of precipitins. A protein fraction separated by urea extraction has been found to sensitize animals,[24] and an effective immunizing preparation of subcellular particulate material has been prepared from virulent human strains, and also from BCG but of much reduced immunizing efficiency, by Youmans and his coworkers.[59, 60]

The hypersensitivity so produced is of the delayed kind typical of infection allergies. It is not passively transferable by serum but may be transferred by cells from a hypersensitive animal.

Tuberculin. The sensitized animal will react to the soluble cell substance of the tubercle bacillus, preparations of which have been called *tuberculin.* Tuberculin is usually prepared from the human type of tubercle bacillus, though bovine tuberculin is practically as active as human tuberculin in infections with the human bacillus; avian tuberculin, although considerably less active, will also produce a reaction. A variety of tuberculins have been prepared of which only a few need be noted here. The first of these was made by Koch, and consisted of the filtrate of a glycerol-broth culture of the bacilli concentrated by evaporation on a water bath to about one tenth its original volume (the activity is heat-stable). This material is "original" or "old" tuberculin (TO or OT). A "new" tuberculin (TR—tuberculin residuum) was prepared by Koch in 1897 by macerating living virulent bacilli and extracting the mass with water, and then making an emulsion of the residuum. He later advocated the use of an emulsion (BE—bacillary emulsion or *Bazillenemulsion*) of the entire substance of young virulent bacilli in 20 per cent glycerol, actually a vaccine. Denys introduced the use of the unaltered filtrate from broth cultures (BF—broth filtrate). None of these later innovations, however, proved to be superior to old tuberculin, and the original preparation, or slight modifications of it, has been widely used.

The active principle of tuberculin is protein in nature, and the cultivation of the tubercle bacillus in glycerol-asparagin-citrate synthetic solutions by Long and Seibert has made possible the study of the active principle in purified preparations. The activity is associated with a number of protein fractions, one of which Seibert prepared in crystalline form. A more satisfactory preparation of low molecular weight, *ca.* 2000, has been isolated by Seibert by precipitation with trichloracetic acid. Originally designated SOTT (synthetic medium old tuberculin trichloracetic acid precipitated), it is now known as PPD (purified protein derivative).[111, 112, 113]

The comparative merits of OT and PPD have been the subject of a series of investigations. OT is relatively unstable in dilutions, while PPD, a dry powder, is "dry-diluted" with lactose and is indefinitely stable in this form. Different lots of OT vary somewhat in their activity; the activity of PPD preparations is relatively constant. It appears that PPD is quite as satisfactory as OT in actual use and, because of its stability and constant activity, is regarded by many as superior to OT.

The tuberculin reaction.[68] Three types of reaction may be elicited in the sensitized, *i.e.,* infected, animal by the injection of tuberculin. In addition to a local inflammatory reaction at the site of inoculation, there is a focal reaction manifested as an acute congestion around tuberculous foci which, if marked, may aggravate the pathological process, and a constitutional reaction in which the temperature rises to a peak of 102° to 104° F. and subsides in 12 to 18 hours. In man the constitutional reaction also includes malaise, pain in the limbs, and, perhaps, vomiting, dyspnea, and other symptoms. These reactions do not appear in normal animals. The utility of tuberculin is, then, twofold; it may be used for diagnostic purposes and it has therapeutic value, though the latter is strictly limited.

The diagnostic tuberculin test in man is generally a skin test. Koch's original method consisted of subcutaneous injection of tuberculin. The cutaneous reaction of von Pirquet involves the rubbing of tuberculin onto the scarified skin. In the Mantoux test, the one most commonly used today, graded doses of tuberculin are injected intradermally, usually starting with 0.01 mg. of OT and going as high as 1.0 mg. or even 10 mg. on rare occasions (0.1 ml. of a 1:100 dilution of OT is supposed to contain 1 mg.; the standardization of new batches is biological and carried out in guinea pigs infected with virulent tubercle bacilli). Smaller amounts of PPD are used, since it is in dry, pure form; usually 0.00005 to 0.005 mg. A "patch test" has been introduced by

Vollmer in which squares (0.8 cm.) of thin filter paper, impregnated with tuberculin about four times as strong as the original old tuberculin and dried, are taped on the cleansed skin over the sternum or upper edge of the trapezius. The patch test appears to be somewhat less sensitive than the intracutaneous test of Mantoux. Since these are all skin tests only the local inflammatory reaction is observed in infected persons. There is evidence, however, that repeated tuberculin testing may result in a local sensitivity in the uninfected person.[36, 90]

In young children a positive tuberculin reaction may be taken as indicative of infection. It was formerly believed that, once established, the hypersensitivity persists essentially throughout life and that the tuberculin reaction is of limited value in the adult. It is becoming apparent, however, that reversion is more frequent than had been generally realized, particularly with reduction in the prevalence of the disease and therefore the risk of reinfection.

The tuberculin test in cattle has great diagnostic importance and has been widely used in the United States and to a lesser extent elsewhere. Three types of test may be used in cattle: the intradermal test; the ophthalmic reaction of Calmette, in which tuberculin is dropped into the conjunctiva and the reactive animal responds with the development of a diffuse congestion and edema in 6 to 8 hours which fades away in 24 to 36 hours; and the constitutional reaction as indicated by a rise in temperature following the injection of tuberculin. The intradermal inoculation into the skin of the caudal fold is generally practiced in this country.

The therapeutic value of tuberculin may be directly observed in lupus or tuberculous infection of the skin. As indicated above, there is a reaction about the foci of infection manifested as acute congestion and sloughing off of tissue. When it was first introduced, tuberculin was regarded by many as a highly effective specific therapeutic agent for tuberculosis. Its use is, however, exceedingly dangerous, and, with the exception of lupus, the response of tuberculous infection to the injection of tuberculin has been disappointing.

The mechanism of immunity. Infection with the tubercle bacillus confers a definite protection against reinfection, an "immunity to superinfection" resembling that observed in syphilis. The factors involved are as yet obscure. Some current opinion favors the view that the development of an allergic state is indicative of an effective immunity. It is commonly observed that in individuals giving a positive tuberculin reaction living cells are usually free of tubercle bacilli and the bacteria are present in necrotic areas separated by an avascular barrier, while in the infected individual giving a negative reaction, tubercle bacilli are found in great numbers in living tissue. The antibodies produced are apparently not significant and antiserums have no protective or curative properties. As indicated above, the development of hypersensitivity is the most obvious response to infection, and there is no doubt that hypersensitivity plays a part in acquired resistance as indicated in Koch's early experiments. Its relative importance is, however, not at all clear, and the mechanism of what seems to be a low-grade immunity of short duration remains at the moment largely a matter of speculation.

Active immunization. The possibility of active immunization to tuberculosis has been of great interest since the discovery of the tubercle bacillus. In general, two types of vaccines have been employed, suspensions of living attenuated bacilli and suspensions of killed bacilli.[110]

The attenuated bovine strain of Calmette, BCG, has been regarded as the most promising immunizing agent and has been extensively studied. It was originally given as an oral vaccine in France in the early 1920's. However, the combination of inadequate statistical data and an incident in Lübeck, Germany, in which a virulent strain was inadvertently substituted for the vaccine strain, resulting in tuberculosis in in-

Figure 180. Avian tubercle bacillus. Acid-fast stain of a smear from a pure culture. × 1050.

oculated persons, put it into disrepute. Nevertheless immunization with BCG was further studied in the Scandinavian countries, beginning in 1925 in Sweden and in 1927 in Norway and Denmark. The freshly prepared vaccines have a relatively short useful life, perhaps 7 to 10 days, but lyophilized vaccines, after an initial loss of some viability, are stable for a year when stored at 20° C. or less.[54, 89, 125] The vaccine is given intracutaneously in doses of 0.05 to 0.15 mg., and a positive tuberculin reaction appears in 6 to 10 weeks in over 90 per cent of those inoculated. The hypersensitivity lasts for about 4 years, though in some persons there is reversion in as little as 1 year. On the assumption that a positive tuberculin reaction is indicative of immunity, reversion is taken to mean that reinoculation is required.

There is a large body of evidence[25, 105] which supports the conclusion that such immunization gives an appreciable degree of protection against childhood tuberculosis, but whether against infection later in life is not so clear. It is believed by some,[86] however, that the immunization, involving the use of a living vaccine whose virulence may not be fixed, is a dangerous one, and that confidence cannot be placed in immune response of the newborn and immature infant; fatal cases of infection with BCG have, in fact, occurred,[80, 131] but it is surprising that there have not been more in view of the many thousands of inoculations that have been carried out.

Immunization has been very generally applied in Scandinavian countries (where its effect on the decline in tuberculosis is probably overestimated), less so in Europe, and in only a restricted way in this country. While a general program of immunization may serve as a control measure in areas in which the disease is widespread and the general application of chemotherapy, isolation, etc., is not practical,[99] it appears to have only limited utility, as in groups exposed to unusual risk of infection, in this country.[44, 98]

Pathogenicity for Lower Animals. Tuberculosis of lower animals living under natural conditions is probably very rare. Animals in captivity, however, may contract the infection with some facility; in animals kept in zoos and in monkeys in the laboratory for experimental purposes tuberculosis is not uncommon. Domestic animals may be similarly affected. A wide variety of animals may be infected experimentally with one or another of the types of tubercle bacilli.

Domestic animals. The most commonly infected domestic animals are cattle, pigs, and chickens. Cattle are infected with the bovine type of tubercle bacillus almost exclusively; they are not completely resistant to the human type, as Koch originally thought, but infection is accomplished with some difficulty. The proportion of cattle infected in-

creases with advancing age and, in the absence of control measures, may reach 70 to 90 and possibly 100 per cent in animals kept in stalls. The natural infection is generally of a chronic, slowly progressive nature. The lymphatics are most frequently involved and may be the only tissues to show lesions, the lungs are also commonly affected. Lesions on the pleura have a peculiar characteristic appearance, the so-called perlsucht disease. The liver, spleen, and kidneys are less frequently involved, infection of the mammary glands is not uncommon, and tubercle bacilli may be excreted in the milk in the absence of detectable lesions of the mammae. Congenital tuberculosis in cattle occurs with some frequency. In this country tuberculous infection in cattle is rigorously controlled; over nine million cattle are tested annually, and the percentage of infected cattle found is 0.19 and of infected herds 1.4 to 1.6.

Tuberculosis in chickens is very common and is exclusively an infection with the avian variety of the tubercle bacillus. With the exception of parrots and certain birds of prey, birds are highly resistant to infection with the human and bovine varieties, and it is probable that natural infection with these types rarely if ever occurs. Tuberculosis in chickens is usually a chronic process and is characterized by the formation of nodules in the abdominal viscera. The lungs are less frequently affected.

Pigs suffer from natural infection with both bovine and avian tubercle bacilli derived from infected cattle and poultry; they are also susceptible to infection with the human variety. In young pigs infection with bovine bacilli is generalized and acute with lesions in the lymphoid tissue, abdominal viscera, and lungs. Infection with the human bacillus is generally localized, but the avian type may produce a generalized infection.

Other domestic animals suffer from tuberculosis to a considerably lesser extent. Horses, dogs, and cats are occasionally infected, and the disease is rare in sheep and goats.

Experimental animals. Experimental animals vary in their susceptibility to the varieties of the tubercle bacillus and in the type of infection produced. The guinea pig is highly susceptible to both bovine and human bacilli, and death follows the subcutaneous injection even of small doses in 6 to 15 weeks. The lymphatic glands, spleen, and liver are most affected, the lungs only slightly, and the kidneys never. The necrotic areas in the spleen and liver are the most striking feature of the gross pathology and are peculiar to the guinea pig. True tubercles are seldom seen except in the very early stages of the disease.

Rabbits are highly susceptible to infection with the bovine bacillus, somewhat so to the avian bacillus, and quite resistant to the human variety. Injection of bovine bacilli produces a generalized infection that terminates fatally in 2 to 3 months. On autopsy tubercles may be found in the spleen and liver, but the lesions are most marked in the lungs and kidneys and may even be confined to them. Very large doses of human bacilli (10 to 50 mg. intraperitoneally) may produce a progressive infection but not the acute fatal miliary disease. The mammalian tubercle bacilli, indistinguishable by cultural or serological methods, may, then, be sharply differen-

Characteristics of the Varieties of Tubercle Bacilli

VARIETY	PHYSIOLOGY				PATHOGENICITY		
	Opt. temp.	Rate of growth	Pigment	Stimulation by glycerol	Guinea pig	Rabbit	Chicken
Human	37° C.	eugonic	+	+	++++	±	–
Bovine	37° C.	dysgonic	–	–	++++	++++	–
Avian	40–42° C.	rapid	+	+	+	++	++++

tiated by their pathogenicity for these two experimental animals.

Neither rabbit nor guinea pig is particularly susceptible to infection with the avian bacillus though avian tuberculosis may be produced in the rabbit.[42] In the guinea pig death may be produced by large intraperitoneal doses, but on autopsy macroscopic tubercles are not visible; cultures and smears of the liver and spleen, however, show the presence of the bacilli. This form of tuberculosis, the proliferation of the bacilli without macroscopic tubercle formation, is known as the Yersin type of tuberculosis.

Experimental infections of mice inoculated with the human variety of tubercle bacilli have been studied at length by Youmans and his coworkers.[94, 137, 140, 142] They have found that the median survival time of the infected animals is a linear function of the logarithm of the dose and that the generation time of the tubercle bacillus is 4 to 6 days *in vivo* in this animal as compared to 14 hours *in vitro*. These precise studies have made the mouse a valuable experimental animal, and it has been used extensively in work on chemotherapeutic drugs.

Epidemiology.[41, 56, 135] In man tuberculosis is largely an air-borne infection. Dissemination by milk is now of relatively minor importance in the United States. The disease is one of civilization in that its transmission is facilitated by close contact. The frequency of positive tuberculin reactions rises rapidly from zero in the newborn through puberty, and many adults have been infected at one time or another. In most instances clinical tuberculosis has not occurred and the lesions have healed. The proportion of persons with active clinical tuberculosis is not known; in some investigations the ratio of cases to deaths has been found to be as high as 10:1 or 12:1, but the ratio of reported cases to deaths is not much more than half of this. The prevalence of tuberculosis, then, defies precise definition.

There are marked racial differences in the prevalence of tuberculosis. The death rate of the Negroes in this country is considerably higher than that of the white population, though the incidence of clinical tuberculosis in the two races is not greatly different. Whether this higher death rate is a consequence of environmental conditions or whether it is in part attributable to racial differences in susceptibility has been a point of considerable interest. In this connection the experience in the United States Army is of particular interest. In the years 1922 to 1936 the average white morbidity rate was 2.10 per 1000 and the Negro rate 2.56, a ratio of 4:5; the white death rate was 0.24 and that of the Negroes 0.99, a ratio of 1:4; and the case-death ratios were 8.75 for the whites and 2.61 for the Negroes. Under the controlled conditions prevailing, *i.e.*, preliminary physical examination, age, and sex selection, the same housing conditions, and identical diagnostic and therapeutic facilities, it would appear that though the incidence of clinical tuberculosis is no greater in the Negro, the disease is more frequently fatal, and the indicated greater susceptibility is a consequence of racial rather than environmental factors. During World War II the Negro, while making up but 10 per cent of the United States Army, contributed 43.4 per cent of the total deaths from tuberculosis. There is some evidence that less well-defined races of man differ in their resistance to tuberculosis; Jews and Italians appear to be more resistant than the Irish.

Tuberculosis has been decreasing at a steady and relatively rapid rate since 1850 or thereabouts as indicated by the decline in the death rate from this disease.[1, 61] As in the case of some other diseases, the decline set in before the discovery of the bacterial etiology of infectious disease and the development of preventive measures and hence is by no means entirely attributable to the practice of preventive medicine.

This decline has not been relatively the same in either the various age groups or in the two sexes. The death rate is highest in the very low age groups, that of 1 to 2 years, and falls rapidly in the 5 to 9 group and then rises to a peak in early adult life, 20 to

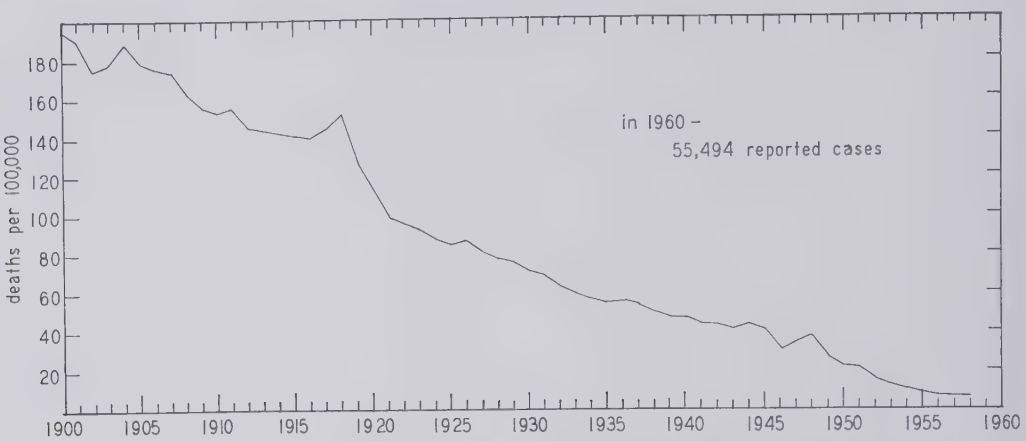


Figure 181. The prevalence of tuberculosis in the Registration Area since 1900 as shown by the mortality rates. Data from the Morbidity and Mortality Weekly Report, vol. 9, 1961. Communicable Disease Center, Public Health Service, U. S. Department of Health, Education and Welfare.

24, and declines with, in recent years, a small secondary peak between 45 and 54 years (late adult tuberculosis). The decline in tuberculosis in the present century has been relatively greatest in the very young, an undoubted consequence of preventive measures.[35]

The sex distribution of the death rates from tuberculosis in the various age groups is a curious phenomenon which has not been explained. The death rate for males of all ages is somewhat higher than that for females. In young adults, *i.e.*, the 15 to 29 age group, the female death rate is considerably higher than the male death rate. In the higher age groups the male death rate becomes proportionately greater and exceeds the female rate for the rest of life. In the present century the female death rate in the higher age groups has declined somewhat more rapidly than the male death rate.

There is no simple explanation for the observed decline in tuberculosis mortality. Probably the isolation of active cases to reduce the source of infection, together with improvement in living conditions, both environmental and nutritional, has played a significant part. Such a decline is usually taken as a manifestation of the inability of a disease to reproduce itself and leads to the prediction of its eventual disappearance. This need not necessarily follow, however, and in this country the number of new cases reported has increased since 1940. For example, between 1940 and 1947 the number of deaths declined by 20 per cent, while the number of new cases reported rose from 100,772 in 1940 to 133,837 in 1947 and then declined to 55,494 in 1960.

In this country, England, and the Scandinavian countries there appears to be a reservoir of minimal undiagnosed active tuberculosis of the late adult type concentrated in the age groups over 45 that is increasing.[84, 103, 134] In New York City, for example, the proportion of new cases and deaths in persons over 45 doubled in the 20-year period prior to 1950, and 65 per cent of all deaths were of persons over 45 of which 85 per cent were male. If this represents a residual of higher rates in earlier life, it will eventually decline, but if it is a consequence of suppression of the disease in otherwise susceptible persons by environmental factors, the reservoir of such persons may increase. It represents a source of infection to a population becoming increasingly more susceptible as a consequence of reduction in immunizing infections.

MYCOBACTERIUM LEPRAE (HANSEN'S BACILLUS)[4, 21, 104]

Like tuberculosis, leprosy is an old disease of man. The first accurate description of the disease in India was in

the Sushruta Samhita about 600 B.C.; it was known in Egypt in the time of the Pharaohs, and in China in the fifth century B.C. Perhaps more prevalent in ancient times, it is now most common in Central Africa, India, Japan, and other Asiatic countries. The disease is prevalent in South America, with endemic centers in Brazil, Colombia, and Argentina. It is estimated[12] that there are 80,000 cases in Brazil, 50,000 in Mexico, 16,000 in Argentina, 12,000 in Colombia, 10,000 in Paraguay, 6,000 in Cuba, and 3,400 in Peru. It is relatively uncommon in Europe, and sporadic cases occur in Latvia, Estonia, southern and eastern Russia, and on the Mediterranean coast. The total number of lepers in the world is not accurately known; estimates vary from two to seven million.

Leprosy has been introduced into the United States with varying consequences. In Louisiana, Florida, and Texas the imported cases have established foci in which the disease has a tendency to perpetuate itself, while in California and the central northwestern states it tends to die out. Elsewhere in the country transmission is so rare as to be negligible.[6] It is estimated that there are 500 to 1000 lepers in this country, many of whom are segregated in the National Leprosarium in Carville, Louisiana, the remainder living for the most part in California and New York.

Bacilli were found by Hansen in 1872 in the round epithelioid cells generally known as lepra cells, and the observation was one of the first of pathogenic bacteria.

Morphology and Staining.[29] Morphologically the leprosy bacilli closely resemble the tubercle bacilli. They are long (6 μ), slender rods, usually straight, but sometimes slightly curved. They are nonmotile and do not produce spores. They generally occur within the cells but are sometimes found free in the lymph spaces. Their arrangement within the cells is characteristic, several bacilli being usually grouped together in bundles like packets of cigarettes. Capsules are present on cells which are presumed to be viable, but are destroyed by the use of hot carbol-fuchsin for staining.[50, 51, 52, 53]

The staining reaction of these microorganisms is much like that of the tubercle bacilli. They stain somewhat more readily than the latter, and also decolorize more quickly with acids, but the difference is not great enough for differentiation. The presence of large numbers of bacilli within the cells, together with the clinical features of the disease, makes it possible to distinguish leprosy bacilli from tubercle bacilli without difficulty. Because of their acid-fast staining characteristic and their morphological resemblance to the tubercle bacilli and similar bacteria, these bacilli are included with the mycobacteria and designated *Mycobacterium leprae.*

Cultivation. Numerous unsuccessful attempts to cultivate Hansen's bacillus on artificial mediums were made for years by bacteriologists all over the world. A few investigators have reported positive results. In most instances acid-fast bacilli have been cultivated, but in others a variety of microorganisms, including diphtheroids, actinomycetes, and anaerobic bacilli, have been found. It has been suggested by some workers that the acid-fast bacilli present in leprous lesions represent but a single stage in a developmental cycle and outside the body other forms appear. There are at present a number of cultures in various laboratories labeled *Myco. leprae.* It seems highly probable that none of these bacteria are leprosy bacilli in that they are etiologically related to the disease in man. They are very likely best grouped with the saprophytic acid-fast forms such as the smegma and timothy bacilli.

Among the more recent studies on the isolation of leprosy bacilli are those of De Souza-Araujo,[27, 28] who has isolated acid-fast bacilli that have produced suggestive reactions on inoculation into monkeys. Freire[45] has cultivated similar bacilli in slide cultures. There have been a number of reports of cultivation, *i.e.*, persistence with apparently a few cell divisions, in tissue culture.[8, 31, 32]

Pathogenicity. Although any organ

Figure 182. The leprosy bacillus. Acid-fast stained smear from a skin lesion. Note the characteristic tendency to parallel arrangement of the bacilli in packets. × 1800.

or tissue in man may be attacked with varying results, two distinct types of leprosy are usually recognized—the nodular and the anesthetic. The former, which is the more acute, is characterized by the development of masses of granulation tissue, the so-called leproma, which may appear superficially in different parts of the body and by their growth and coalescence produce distortion and mutilation. The anesthetic type, or nerve leprosy, progresses more slowly, the average duration of the cases being nearly twice as long (18 years) as cases of the nodular type, some being known to extend over 35 or 40 years. Atrophy of the muscles and other trophic disturbances accompany the nerve lesions.

In both forms of leprosy Hansen's bacillus is found in all cases, in enormous numbers as a rule in the lesions of nodular leprosy and less abundantly in the anesthetic type. Very few bacilli are observed outside the body cells, and they are found in the cytoplasm and do not invade the nucleus. Almost any part of the body may be the site of leprous growth; the kidneys are usually invaded, the liver and spleen always. The bacilli have been seen in the central nervous system and are occasionally found in the blood, generally in the leucocytes but sometimes free. There are a number of classifications of the clinical disease.[19]

Relative Proportion of Types of Leprosy*

REGION	NEURAL TYPE %	LEPROMATOUS TYPE %
Africa	90.5	9.5
Philippines	50.0	50.0
Mexico	40.0	60.0
Java	29.0	71.0

* Data from Lowe: Proc. Sixth Pacific Sci. Congr., 1942, 5:921.

Leprosy has never been unequivocally produced in experimental animals by the inoculation of leprous material from man. The inoculation of relatively large amounts of lepromatous material to produce a localized granulomatous cellular response has been described several times,[13, 78] and in some instances it has been possible, in mice, to pass the reaction three or four times.[117, 118]

Transmission. Leprosy is probably transmitted by contact, though the conditions that make transmission possible are entirely unknown. There are numerous instances in which healthy persons, such as asylum attendants, have been more or less in contact with lepers for long periods without contracting the disease. In other cases, however, leprosy has been contracted by those in close and long-continued contact with diseased individuals. There is also indirect evidence of transmission. Manson cites the case of an Irishman who acquired leprosy in the West Indies. On his re-

turn to Ireland his bed was shared by his brother who, moreover, sometimes wore the leper's clothes. The brother, who had never been in any foreign country, became in time an undoubted leper. In this instance communication from one person to another was practically demonstrated. A large percentage of cases studied in Hawaii gave a history of exposure, and usually such exposure was of an intimate character. Leprosy occurs for the most part in populations of low social order living under unsanitary conditions of poverty, crowding, uncleanliness, and sickness. Even so, a relatively small proportion of those exposed develop the disease. Age at the time of exposure is important, the risk being greatest for those exposed before 5 years of age. Of children of lepers, separated from their parents and who develop leprosy subsequently, the disease appears within the first year in 50 per cent, and in 90 per cent within 3 years, after the last exposure.[91]

One way in which the bacillus may leave the body is in the nasal mucus, and large numbers of the bacilli are found in the secretions of the nose in many cases. Bacilli may sometimes be discharged from the mouth or nose in small particles of mucus. The demonstration of typical acid-fast bacilli, often in very large numbers, in nasal smears or in biopsy specimens[49, 62, 65, 70] constitutes the laboratory diagnosis of the disease. In the opinion of many writers, the mucous membrane of the nasopharynx is the point at which the bacteria are introduced into the body, as well as the chief source from which infection is spread.

Evidence of the direct inoculability of leprosy from man to man is quite inadequate. Many attempts to infect healthy persons have been made and have failed, and one often cited instance of successful inoculation is by no means unimpeachable. In the case of the criminal Keanu in the Hawaiian Islands, reported by Arning in 1893, implantation of material from a leprosy nodule was followed by the development of true leprosy, which terminated fatally 6 years after inoculation. The experiment, however, did not include the important source of error involved in the facts that Keanu was a native of a country in which leprosy was common, that he had lived among lepers, and that members of his family were lepers. Later Lagoudaky[65] inoculated himself intramuscularly with blood from two lepers and contracted the disease.

Much light is thrown on the contagious character of leprosy by the success that has attended the isolation and segregation of leprous patients. The Norwegian experience showed that a careful but not unduly rigorous system of separation was accompanied by a diminution of the number of cases from 2870 in 1856 to 577 in 1900 and to 7 in 1957.[128] The circumstance that infection does not invariably follow chance contact or association should not, therefore, lead to neglect of the facts that leprosy is a bacterial disease; that up to the present under natural conditions the specific bacterium has not been found except in the human body; and that, so far as is definitely known, the leper himself is the most important means by which leprosy spreads.

Chemotherapy.[20, 33] The similarities between the acid-fast bacilli observed in leprosy and the tubercle bacillus inevitably suggested use of the drugs effective in tuberculosis in the chemotherapy of leprosy. Of these, the sulfones have been the most satisfactory, usually giving relatively rapid clinical improvement and slower bacteriological improvement. There is some tendency to relapse following cessation of chemotherapy. Diaminodiphenyl sulfone (DDS, Dapsone) is considered to be the chemotherapeutic agent of choice,[47] and the activity of various derivatives seems to be attributable to the extent to which degradation to the parent compound occurs *in vivo*. The thiosemicarbazones appear to be reasonably effective but less so than the sulfones and are regarded as useful as an alternative when sulfones are not tolerated. Para-aminosalicylic acid has little chemotherapeutic activity and preliminary studies with isonicotinic acid hydrazide have not been encouraging. Streptomycin has appreci-

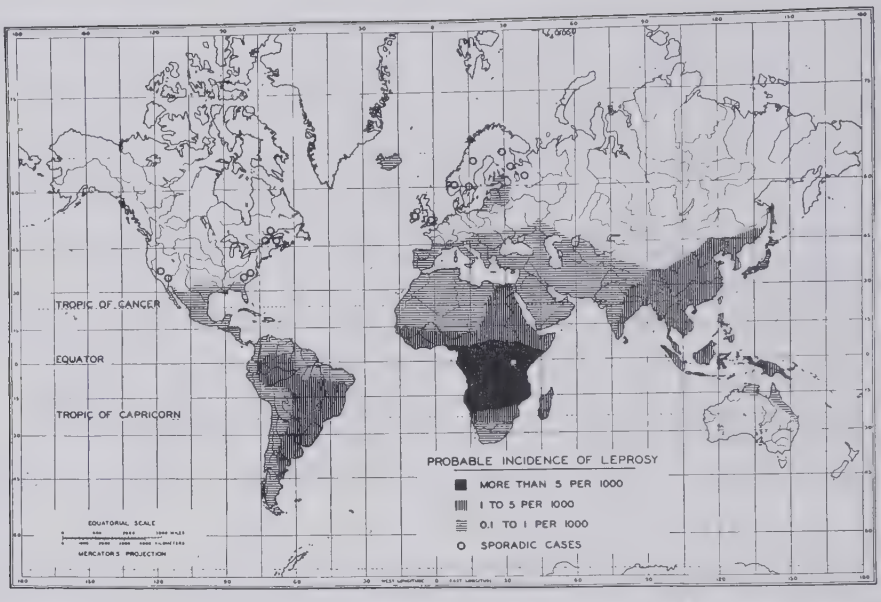


Figure 183. The probable prevalence of leprosy in the world as indicated by present data. Based on Goode *Base Map* No. 201 M. By permission of the University of Chicago Press. (After Saunders.)

able chemotherapeutic activity but has been found too toxic for the long period of administration required.

Immunity. Man is highly resistant to infection with the leprosy bacillus, and, after infection is established, the ensuing disease is essentially a benign process that may take many years to terminate fatally; in fact, many lepers die from other causes. Little is known regarding the specific immune response. Lepers do, however, develop a hypersensitivity to the cell substance of acid-fast bacilli such as the tubercle bacillus and the various saprophytic species. Muir[85] has suggested that the acid-fast bacilli exist as a continuous series with respect to parasitism, ranging from the saprophytic forms through the chromogenic forms occasionally found associated with the disease, the tubercle bacilli of clear host specificity, to the leprosy bacillus which appears to be unable to live outside the host tissue. A curious fact is the appearance of a positive Wassermann reaction in lepers in the absence of syphilis, and both the Wassermann and the Kahn tests may be positive in lepers having no clinical symptoms of syphilis.

Lepromin.[122] The hypersensitivity developed by the leper may be demonstrated by the intradermal inoculation of material prepared from leprous nodules. Two reactions are observed, one early and occurring after 3 or 4 days, and the late reaction appearing 3 to 4 weeks after inoculation. The first is the *Fernandez reaction* and the second the *Mitsuda reaction.* The Mitsuda reaction is regarded by some workers as an expression of immunity, but it is nonspecific in that it occurs in tuberculosis and is induced by immunization with BCG.

MYCOBACTERIUM LEPRAE MURIUM

A native disease of wild rats, commonly known as rat leprosy, and characterized by enormous numbers of acid-fast bacilli present in the lesions, was described by Stefansky in 1903 as occurring in wild rats in Odessa. The disease was observed in the same year by Dean, who later showed it to be transmissible. It has since been observed in wild rats all over the world.[43]

The acid-fast bacilli closely resemble the leprosy bacillus in size and shape and are found intracellularly but not as often

in the packet arrangement of parallel bacilli.[17, 30] *Myco. leprae murium* has not been cultivated on artificial mediums but has been reported[46, 55, 129] to persist through a few cell divisions in tissue culture. The disease may, however, be transmitted to white rats, mice, and guinea pigs by inoculation with pieces of tissue. Wild mice are relatively nonsusceptible, and subcutaneous inoculation results in only a transient granuloma.[88] The experimental infection is a relatively benign process. A local, circumscribed lesion develops following subcutaneous inoculation which becomes palpable in 4 to 5 weeks and eventually develops to a large tumorous mass ulcerating on the surface and persisting throughout the life of the rat. The earliest lesions in the other organs do not appear before 4 to 6 months, and the animal dies only after a year or more.[16]

The relation of rat leprosy to human leprosy is not known. Rats are not susceptible to inoculation with human leprous material.

OTHER ACID-FAST BACILLI

Mycobacterium Paratuberculosis. A chronic enteritis of cattle usually terminating fatally is caused by an acid-fast bacillus closely resembling the avian variety of the tubercle bacillus. The disease is sometimes called Johne's disease and the bacillus Johne's bacillus after its discoverer. The disease only remotely resembles tuberculous infection. The lesions in the intestinal wall are proliferative, and the granulomatous tissue may contain epithelioid cells and occasionally giant cells, but there is no caseation.

The disease appears to be widespread in the United States. Infected cattle become hypersensitive to the bacillary substance, and filtrates of cultures produce a skin reaction analogous to the tuberculin reaction which is designated the "johnin reaction." No case of human infection with *Myco. paratuberculosis* has been recorded.

The Vole Bacillus. An acid-fast bacillus responsible for an epizootic, chronic infection of the field vole, *Microtus agrestis,* resembling tuberculosis was discovered by Wells in 1937. It closely resembles the tubercle bacillus culturally though it forms no pigment and growth is not enhanced by glycerol. It is pathogenic for both guinea pigs and rabbits, considerably more so for the latter, and is not pathogenic for fowls. It has been suggested that it is a distinct type of mammalian tubercle bacillus and should be called *Myco. tuberculosis* var. *muris.* This microorganism has been of particular interest because, though it produces only a localized and retrogressive infection when inoculated in small doses in guinea pigs and calves, tuberculin sensitivity is produced, and preliminary experiments on its use as a prophylactic have given suggestive results.

"Anonymous" Mycobacteria. [18, 57] While acid-fast bacilli other than tubercle bacilli have been found in association with human disease from time to time, they have been isolated more and more often within the past few years and have attracted increased attention. Because they are not identifiable with known species, they have been known as the "anonymous," "atypical" or "unclassified" mycobacteria.[79]

These bacteria are found in man in the absence of disease, admixed with tubercle bacilli in tuberculous infections, and in large numbers in pure culture in apparently etiologic relation to tuberculous-like disease. The pulmonary disease[11, 66] closely simulates tuberculosis, but endobronchitis seems to be more common, and there is somewhat more nonspecific inflammation and fibrosis. Similarly, the pathology of lymphatic infection resembles that of tuberculous infection, including calcification in the later stages, but the infected nodes tend more often to be necrotic and suppurative in character. These infections are not common, and are reported to make up 1 per cent or less of suspected tuberculosis.

These bacteria differ from the human tubercle bacilli in that they are not pathogenic for the guinea pig, are relatively

resistant to the antituberculous chemotherapeutic agents, grow more rapidly, and some are pigmented.[74, 75] They are separated by Runyon into four groups, *viz.*:

Chromogens:

Group I. Photochromogens, producing a bright yellow pigment in the presence of light; many strains are pathogenic for man and the mouse; includes *Myco. kansasii* (*Myco. luciflavum*).

Group II. Skotochromogens (scotochromogens), producing a reddish-orange pigment independent of light; usually nonpathogenic for man or the mouse; includes *Myco. scrofulaceum.*

Nonchromogens:

Group III. Filamentous forms, possibly including some Nocardia strains; some strains pathogenic for man and the mouse; includes the Battey bacillus and *Myco.* (*Nocardia*) *intracellularis.*

Group IV. The "rapid growers," characterized by very rapid (2–4 days) growth; a few strains pathogenic for man and the mouse; includes *Myco. fortuitum.*

These bacteria are immunologically related to the tubercle bacilli and other mycobacteria,[9, 143] and are considered by some[136] to have arisen from the former.

"Cold-Blooded" Mycobacteria. Acid-fast bacilli have been found associated with pathological processes in various cold-blooded animals. In some instances the processes superficially resemble tuberculous lesions. *Myco. piscium* was isolated from nodules and tumor-like formations in carp; *Myco. marinum* from "tuberculosis" of sea bass and certain other salt-water fish; *Myco. ranae* was found in the liver of a frog; *Myco. thamnopheos* is a parasite in garter snakes; and *Myco. chelonei* is the so-called turtle bacillus.

Saprophytic Acid-Fast Bacilli. Included in this category are the well known timothy bacillus, *Myco. phlei,* found in soil, on grasses, and elsewhere in nature; the "butter bacillus," *Myco. butyricum;* and *Myco. smegmatis,* which is, however, a parasite found in both male and female smegma. The smegma bacillus is often difficult to distinguish from the tubercle bacillus on morphological grounds, and confusion of the two may have considerable practical importance in the diagnosis of suspected cases of tuberculous infection of the urinary tract. It is, it may be noted, also found in the urine and may contaminate fecal specimens. The saprophytic bacilli all grow much more rapidly than the tubercle bacilli, and neither they nor the bacilli isolated from cold-blooded animals are pathogenic for guinea pigs and rabbits, or at best only feebly so.

REFERENCES

1. Anderson, G. W.: Epidemiology of tuberculosis. Amer. Rev. Tuberc., 1953, **67:**123–131.
2. Anderson, R. J.: Chemistry of lipoids of tubercle bacilli. Physiol. Rev., 1932, **12:**166–189.
3. Anderson, R. J.: Structural peculiarities of acid-fast bacterial lipids. Chem. Rev., 1941, **29:** 225–243.
4. Arnold, H. L.: Modern Concepts of Leprosy. Thomas, Springfield, Illinois. 1953.
5. Auerbach, O.: Pulmonary tuberculosis after the prolonged use of chemotherapy. Amer. Rev. Tuberc., 1955, **71:**165–185.
6. Badger, L. F.: History of leprosy in the United States. Ann. N. Y. Acad. Sci., 1951, **54:**6–11.
7. Bassermann, F. J.: Probleme der Morphologie, Cytochemie und Wuchsform des Tuberkuloseerregers. Thieme, Stuttgart. 1953.
8. Baylet, R., Camain, R., and Basset, A.: Tentative de culture de bacilles de Hansen sur cellules de rein de singe. Bull. Soc. Path. Exot., 1960, **53:**836–841.
9. Beck, A.: Serological investigations of atypical acid-fast bacilli. J. Path. Bact., 1961, **82:**45–51.
10. Berthrong, M., and Hamilton, M. A.: Tissue culture studies on resistance in tuberculosis. I. Normal guinea pig monocytes with tubercle bacilli of different virulence. Amer. Rev. Tuberc., 1958, **77:**436–449.
11. Bialkin, G., Pollak, A., and Weil, A. J.: Pulmonary infection with *Mycobacterium kansasii.* Amer. J. Dis. Children, 1961, **101:** 739–748.
12. Bica, A. N., Roman, J., and Sáenz, A. C.: El problema de la lepra en las Américas. Biol. Oficina Sanitaria Panamericana, 1957, **42:** 548–556.
13. Binford, C. H.: Histiocytic granulomatous mycobacterial lesions produced in the golden hamster (*Crecetus auratus*) inoculated with human leprosy. Negative results in ten experiments using other animals. Internat. J. Leprosy, 1958, **26:**318–324.
14. Bloch, H.: Acid-fast bacteria. Ann. Rev. Microbiol., 1953, **7:**19–46.
15. Bloch, H.: The biochemical properties of virulent and avirulent strains of *Mycobacterium*

tuberculosis. Ann. N. Y. Acad. Sci., 1960, **88:**1075–1086.
16. Chang, Y. T.: Evolution of murine leprosy. Amer. Rev. Tuberc., 1959, **79:**805–809.
17. Chapman, G. B., Hanks, J. H., and Wallace, J. H.: An electron microscope study of the disposition and fine structure of *Mycobacterium leprae-murium* in mouse spleen. J. Bact., 1959, **77:**205–211.
18. Chapman, J. S. (ed.): The Anonymous Mycobacteria in Human Disease. Thomas, Springfield, Illinois. 1960.
19. Chaussinand, R.: Classification of leprosy. Leprosy Rev., 1961, **32:**74–81.
20. Cochrane, R. G.: The treatment of leprosy. Arch. Intern. Med., 1956, **97:**208–214.
21. Cochrane, R. G.: Leprosy in Theory and Practice. Stonebridge Press, Bristol. 1959.
22. Cohn, M. L., Middlebrook, G., and Russell, W. F., Jr.: Combined drug treatment of tuberculosis. I. Prevention of emergence of mutant populations of tubercle bacilli resistant to both streptomycin and isoniazid in vitro. J. Clin. Invest., 1959, **38:**1349–1355.
23. Corper, H. J., and Nelson, C. R.: Methods of concentrating acid-fast bacilli. Amer. J. Clin. Path., 1949, **19:**269–273.
24. Crowle, A. J.: Immunizing constituents of the tubercle bacillus. Bact. Rev., 1958, **22:**183–203.
25. Dahlström, G.: The future of BCG vaccination. Acta Tuberc. Scand., 1957, **32:**250–259.
26. Darzins, E.: The Bacteriology of Tuberculosis. University of Minnesota Press, 1958.
27. De Souza-Araujo, H. C.: Demonstration of two new cultures of *Mycobacterium leprae hominis* (strains "Chaves" and "Emilia") pathogenic for *Macacus rhesus* and man. Trans. Roy. Soc. Trop. Med. Hyg., 1951, **54:** 151–154.
28. De Souza-Araujo, H. C.: A nonchromogenic culture of an acid-fast bacillus isolated from the nasal mucus of a leprosy patient. Its virulence for laboratory animals. Internat. J. Leprosy, 1952, **20:**355–360.
29. De Souza-Araujo, H. C.: The morphology of *Mycobacterium leprae*. Leprosy Rev., 1959, **30:**80–84.
30. De Souza-Araujo, H. C.: I. Bacteriology of rat leprosy: Electron micrographs of rat lepromas and cultures. II. Bacteriology of human leprosy. Leprosy Rev., 1960, **31:**178–188.
31. De Souza, P. R., De Azevedo, M. P., and De Castro, M. P.: *Mycobacterium leprae* em cultura de tecido. Rev. Assn. Med. Brasileira, 1959, **5:**408–412.
32. Devignat, R.: Multiplication of Hansen's bacillus in complex symbiosis in vitro. Nature, 1961, **190:**832.
33. Doull, J. A.: Current status of the therapy of leprosy. J. Amer. Med. Assn., 1960, **173:**363–373.
34. Dragsted, I.: Avian tuberculosis in man. Lancet, 1949, **ii:**103–105.
35. Dublin, L. I.: The course of tuberculosis mortality and morbidity in the United States. Amer. J. Pub. Health, 1958, **48:**1439–1448.
36. Duboczy, B. O., and Brown, B. T.: Local sensitization to tuberculin. Amer. Rev. Resp. Dis., 1961, **84:**69–77.
37. Dubos, R. J.: Rapid and submerged growth of mycobacteria in liquid media. Proc. Soc. Exptl. Biol. Med., 1945, **58:**361–362.
38. Dubos, R. J.: Properties and structures of tubercle bacilli concerned in their pathogenicity. Society for General Microbiology, Symposium No. 5, pp. 103–125. Cambridge University Press, 1955.
39. Dubos, R. J., and Davis, B. D.: Factors affecting the growth of tubercle bacilli in liquid media. J. Exptl. Med., 1946, **83:**409–423.
40. Dubos, R. J., and Middlebrook, G.: Media for tubercle bacilli. Amer. Rev. Tuberc., 1947, **56:**334–345.
41. Feldmann, F. M.: How much control of tuberculosis: 1937–1957–1977? Amer. J. Pub. Health, 1957, **47:**1235–1241.
42. Feldman, W. H.: Avian Tuberculosis Infection. Williams and Wilkins, Baltimore. 1943.
43. Fielding, J. W.: Rat leprosy: Observations and transmission. Med. J. Australia, 1945, **32:** 473–486.
44. Finland, M.: Tuberculosis mortality and morbidity and the problem of BCG vaccination in the United States. New England J. Med., 1959, **261:**699–702.
45. Freire, S. A.: Methods of cultivation of the Hansen bacillus. Slide culture; hemolysis-tube culture; direct inoculation of the liquid medium. Internat. J. Leprosy, 1956, **24:**57–64.
46. Garbutt, E. W., Rees, R. J. W., and Barr, Y. M.: Multiplication of rat-leprosy bacilli in cultures of rat fibroblasts. Lancet, 1958, **ii:**127–128.
47. Garrett, A. S.: Five years of mass Dapsone (DDS) treatment. Leprosy Rev., 1956, **27:** 54–60.
48. Gillis, S.: Some aspects of drug resistance in tuberculosis and its treatment. Med. J. Australia, 1960, **1:**1014–1019.
49. de Grauwe, J., and Verdin, G.: L'interet de la biopsie cutanée dans le diagnostic de la lepre et le controle des resultats thérapeutiques. Quelques considerations sur l'évolution de la lapre traitée. Ann. Soc. Belge Med. Trop., 1957, **37:**443–457.
50. Hanks, J. H.: Significance of capsular components of *Mycobacterium leprae* and other mycobacteria. Internat. J. Leprosy, 1961, **29:** 74–83.
51. Hanks, J. H.: The origin of the capsules on *Mycobacterium leprae* and other tissue-grown mycobacteria. Internat. J. Leprosy, 1961, **29:**172–174.
52. Hanks, J. H.: The problem of preserving internal structures in pathogenic mycobacteria by conventional methods of fixation. Internat. J. Leprosy, 1961, **29:**175–178.
53. Hanks, J. H.: Demonstration of capsules on *M. leprae* during carbol-fuchsin staining mechanism of the Ziehl-Neelsen stain. Internat. J. Leprosy, 1961, **29:**179–182.
54. Hauduroy, P., and Tanner, F.: Essais de lyophilisation du vaccine B.C.G. Rev. d'Immunol., 1954, **18:**356–371.
55. Hawking, F.: Growth of rat leprosy bacillus in tissue culture. Leprosy Rev., 1958, **29:**71–80.
56. Heaf, F.: The new epidemiology of tuberculosis. Med. Officer, 1959, **102:**71–75.
57. Hedvall, E.: Atypical acid-fast bacilli. Acta Tuberc. Scand., 1960, **38:**248–260.
58. Jones, O. R., Platt, W. D., and Amill, L. A.: Miliary tuberculosis caused by intravenous self-injection of tubercle bacilli treated successfully with streptomycin therapy. Amer. Rev. Tuberc., 1949, **60:**514–519.

59. Kanai, K., and Youmans, G. P.: Immunogenicity of intracellular particles and cell walls from *Mycobacterium tuberculosis*. J. Bact., 1960, **80:**607–614.
60. Kanai, K., Youmans, G. P., and Youmans, A. S.: Allergenicity of intracellular particles, cell walls and cytoplasmic fluid from *Mycobacterium tuberculosis*. J. Bact., 1960, **80:** 615–621.
61. Katz, J., and Kunofsky, S.: Trends of tuberculosis morbidity and mortality. Amer. Rev. Resp. Dis., 1961, **84:**217–225.
62. Khanolkar, V. R.: Diagnosis of leprosy. Leprosy Rev., 1961, **32:**158–166.
63. Kubica, G. P., and Vestal, A. N.: A rapid drug-susceptibility test for mycobacteria. Preliminary report. Amer. Rev. Resp. Dis., 1961, **83:**593–595.
64. Lagoudaky, S.: Self-inoculation of leprosy—second communication. J. Trop. Med. Hyg., 1937, **40:**77–79.
65. Lew, J., and Chung, M.: The bacteriologic diagnosis of early leprosy by the trypsin digestion method. Internat. J. Leprosy, 1959, **27:**360–365.
66. Lewis, A. G., Jr., et al.: Chronic pulmonary disease due to atypical mycobacterial infections. Amer. Rev. Resp. Dis., 1959, **80:**188–199.
67. Lind, H. E.: Limitations of fluorescent microscopy for detection of acid-fast bacilli. Amer. J. Clin. Path., 1949, **19:**72–75.
68. Long, E. R.: The tuberculin test. Its value and its limitations. Amer. Rev. Tuberc., 1939, **40:**607–620.
69. Loring, W. E., and Vandiviere, H. M.: The treated pulmonary lesion and its tubercle bacillus. I. Pathology and pathogenesis. Amer. J. Med. Sci., 1956, **232:**20–29.
70. Lowry, L.: Processing of biopsies for leprosy bacilli. J. Med. Lab. Tech., 1956, **13:**558–560.
71. Mackaness, G. B., Smith, N., and Wells, A. Q.: The growth of intracellular tubercle bacilli in relation to their virulence. Amer. Rev. Tuberc., 1954, **69:**479–494.
72. Mandel, W., Heaton, A. D., Russell, W. F., Jr., and Middlebrook, G.: Combined drug treatment of tuberculosis. II. Studies of antimicrobially-active isoniazid and streptomycin serum levels in adult tuberculous patients. J. Clin. Invest., 1959, **38:**1356–1365.
73. Marks, J.: The design of sensitivity tests on tubercle bacilli. Tubercle, 1961, **42:**314–316.
74. Marks, J., and Trollope, D. R.: A study of the "anonymous" mycobacteria. II. Drug sensitivity; pathogenecity; hypersensitivity. Tubercle, 1960, **41:**127–132.
75. Marks, J., and Trollope, D. R.: A study of the "anonymous" mycobacteria. III. Problems of classification and diagnosis; practical recommendations. Tubercle, 1960, **41:**133–142.
76. Mattman, L. H., Tunstall, L. H., Mathews, W. S., and Gordon, D. L.: L variation in mycobacteria. Amer. Rev. Resp. Dis., 1960, **82:**202–211.
77. McDermott, W.: Antimicrobial therapy of pulmonary tuberculosis. Bull. W. H. O., 1960, **23:**427–461.
78. McFadzean, J. A., and Ridley, D. S.: Studies on the inoculation of *Mycobacterium leprae* into monkeys. Trans. Roy. Soc. Trop. Med. Hyg., 1961, **55:**235–238.
79. McMillen, S., and Kushner, D. S.: Atypical acid-fast bacilli. III. An expanded scheme. Amer. Rev. Resp. Dis., 1959, **80:**434–437.
80. Meyer, J.: A fatal case of tuberculosis produced by BCG. Amer. Rev. Tuberc., 1954, **70:**402–412.
81. Middlebrook, G.: The hemagglutination test in tuberculosis. Amer. Rev. Tuberc., 1950, **62:** 223–226.
82. Middlebrook, G., and Cohn, M. L.: Bacteriology of tuberculosis: Laboratory methods. Amer. J. Pub. Health, 1958, **48:**844–853.
83. Mitchison, D. A.: Virulence of isoniazid-resistant tubercle bacilli. Amer. Rev. Tuberc., 1954, **69:**640–641.
84. Monk, M. A., and Terris, M.: Increase of tuberculosis mortality in elderly men from 1940 to 1950. Amer. J. Pub. Health, 1958, **48:**1020–1030.
85. Muir, E.: Relationship of leprosy to tuberculosis. Leprosy Rev., 1957, **28:**11–19.
86. Myers, J. A.: Immunity in tuberculosis. J. Amer. Med. Assn., 1951, **146:**1492–1500.
87. Nassal, J.: Die ätiologische und epidemiologische Rolle des bovinen und aviären Erregertyps bei der Tuberkulose des Menschen. Deut. med. Woch., 1961, **86:**1855–1861; 1890.
88. Nishimura, S.: Susceptibility of wild rodents to the murine leprosy bacillus. Internat. J. Leprosy, 1960, **28:**428–440.
89. Obayshi, Y.: Dried BCG vaccine. W. H. O. Monograph Series No. 28. World Health Organization, Palais des Nations, Geneva. 1955.
90. O'Grady, F.: Tuberculin sensitization in man. Brit. J. Tuberc., 1957, **51:**74–82.
91. Patel, T. B., Kapoor, P., and Rao, V. N.: Infectivity of leprosy in man. An epidemiological investigation of 1,437 leprosy cases under treatment in Vairag and Savda Leprosy Subsidiary Centres of Sholapur and East Khandesh districts in Maharashtra State. Indian J. Med. Sci., 1961, **15:**529–544.
92. Public Health Laboratory Service: Drug resistance in untreated tuberculosis in England and Wales during 1960. A survey. Tubercle, 1961, **42:**308–313.
93. Raffel, S.: The components of the tubercle bacillus responsible for the delayed type of "infectious" allergy. J. Infect. Dis., 1948, **82:** 267–293.
94. Raleigh, G. W., and Youmans, G. P.: The use of mice in experimental chemotherapy of tuberculosis. I. Rationale and review of the literature. J. Infect. Dis., 1948, **82:**197–204.
95. Raleigh, G. W., and Youmans, G. P.: The use of mice in experimental chemotherapy of tuberculosis. II. Pathology and pathogenesis. J. Infect. Dis., 1948, **82:**205–220.
96. Reed, R. W., and Morgante, O.: Recent advances in the laboratory diagnosis of tuberculosis. Amer. J. Med. Sci., 1956, **231:**320–337.
97. Reinisch, E. H., and Kaufmann, W.: A study of prolonged incubation of cultures for *Mycobacterium tuberculosis*. Amer. Rev. Resp. Dis., 1961, **84:**451–453.
98. Report: Report of ad hoc advisory committee on BCG to the Surgeon General of the United States Public Health Service. Amer. Rev. Tuberc., 1957, **76:**726–731.
99. Report: Review of BCG vaccination programmes. Preliminary report by the Direc-

tor-General. Official Records, W. H. O. No. 96. World Health Organization, Palais des Nations, Geneva. 1959.
100. Report: Tuberculosis laboratory methods for the Veterans Administration—Armed Forces Cooperative Study on the chemotherapy of tuberculosis. Department of Medicine and Surgery, U. S. Veterans Administration. 1960.
101. Riley, R. L.: Aerial dissemination of pulmonary tuberculosis. Amer. Rev. Tuberc., 1957, **76:** 931–941.
102. Riley, R. L.: Airborne pulmonary tuberculosis. Bact. Rev., 1961, **25:**243–248.
103. Robins, A. B.: The age relationship of cases of pulmonary tuberculosis and their associates. Amer. J. Pub. Health, 1953, **43:**718–728.
104. Rogers, L., and Muir, E.: Leprosy. Williams and Wilkins, Baltimore. 1940.
105. Rosenthal, S. R.: Standardization and efficacy of BCG vaccination against tuberculosis. Twenty year study: A critical evaluation. J. Amer. Med. Assn., 1955, **157:**801–807.
106. Sabin, F. R.: Cellular reactions to fractions isolated from tubercle bacilli. Physiol. Rev., 1932, **12:**141–165.
107. Sabin, F. R.: Cellular reactions to fractions from tubercle bacilli. Amer. Rev. Tuberc., 1941, **44:**415–423.
108. Schaeffer, W. B.: Growth requirements of dysgonic and eugonic strains of *Mycobacterium tuberculosis* var. *bovis*. J. Exptl. Med., 1952, **96:**207–219.
109. Schellenberg, H.: Tuberkelbakterien in HeLa-Zellen und anderen Geweben. Ztschr. f. Hyg. u. Infektionskr., 1958, **145:**339–354.
110. Seagle, J. B.: A review of BCG and other antituberculosis vaccines. J. Pediatrics, 1955, **46:** 337–347.
111. Seibert, F. B.: History of the development of purified protein derivative tuberculin. Amer. Rev. Tuberc., 1941, **44:**1–8.
112. Seibert, F. B.: The chemistry of the proteins of the acid-fast bacilli. Bact. Rev., 1941, **5:**69–95.
113. Seibert, F. B.: The chemistry of tuberculin. Chem. Rev., 1944, **34:**107–127.
114. Seibert, F. B.: A theory of immunity in tuberculosis. Perspect. Biol. Med., 1960, **3:**264–281.
115. Shepard, C. C.: Growth characteristics of tubercle bacilli and certain other mycobacteria in HeLa cells. J. Exptl. Med., 1957, **105:**39–48.
116. Shepard, C. C.: A study of the growth in HeLa cells of tubercle bacilli from human sputum. Amer. Rev. Tuberc., 1958, **77:**423–435.
117. Shepard, C. C.: Acid-fast bacilli in nasal secretions in leprosy, and results of inoculation of mice. Amer. J. Hyg., 1960, **71:**147–157.
118. Shepard, C. C.: The experimental disease that follows the injection of human leprosy bacilli into foot-pads of mice. J. Exptl. Med.. 1960, **112:**445–454.
119. Smith, C. M.: The control of bovine tuberculosis. A survey of the present position. Med. Officer, 1958, **99:**45–47.
120. Spitznagel, J. K., and Dubos, R. J.: A fraction of tubercle bacilli possessing primary toxicity. J. Exptl. Med., 1955, **101:**291–311.
121. Sweeney, E. E., and Jann, G. J.: Carbohydrate typing of mycobacteria. Proc. Soc. Exptl. Biol. Med., 1961, **108:**671–674.
122. Taylor, C. E., et al.: The antigenic components of lepromin as assayed in guinea-pigs. Internat. J. Leprosy, 1960, **28:**284–299.
123. Thomas, O. F., Borthwick, W. M., Horne, N. W., and Crofton, J. W.: Infection with drug-resistant tubercle bacilli. Lancet, 1954, **ii:**1308–1310.
124. Tysarowski, W., and Kwiek, S.: Difference between the catalases of acid-fast bacilli. Nature, 1959, **184:**1513–1514.
125. Ungar, G., Farmer, P., and Muggleton, P. W.: Freeze-dried BCG vaccine. Methods adopted in preparation of a standard product. Brit. Med. J., 1956, **ii:**568–571.
126. Vandiviere, H. M., Melvin, I. G., and Kennedy, H. E.: Titration of cord formation as a measure of pathogenicity of wild-type acid-fast bacilli, typical and atypical. Amer. Rev. Tuberc., 1958, **78:**799–801.
127. Vandiviere, H. M., Rogers, C. O., Melvin, I. G., and Willis, H. S.: A rapid method of testing the susceptibility of mycobacteria to antituberculous drugs. Amer. Rev. Resp. Dis., 1961, **84:**399–406.
128. Vogelsang, T. M.: The termination of leprosy in Norway. Internat. J. Leprosy, 1957, **25:** 345–350.
129. Wallace, J. H., Elek, S. D., and Hanks, J. H.: Limited multiplication of *Mycobacterium lepraemurium* in cell cultures. Proc. Soc. Exptl. Biol. Med., 1958, **97:**101–104.
130. Wallgren, A.: Immunity in tuberculosis. Acta Tuberc. Scand., 1953, **28:**155–194.
131. Wallgren, A.: Einige Probleme der Calmetteschen Impfung. Deut. med. Woch., 1961, **86:**105–111.
132. Wayl, P., and Stein, O.: Congenital tuberculosis. Tubercle, 1958, **39:**166–168.
133. Wier, J. A.: Present status of the treatment of tuberculosis. J. Amer. Med. Assn., 1956, **162:** 471–473.
134. Wilkins, E. G., Incidence and onset of pulmonary tuberculosis in old men. Brit. Med. J., 1956, **i:**883–886.
135. Woodruff, C. E.: Remarks on the epidemiology of tuberculosis. A review. Amer. Rev. Tuberc., 1957, **75:**975–986.
136. Xalabarder, C.: The so-called problem of unclassified mycobacteria. Amer. Rev. Resp. Dis., 1961, **83:**1–15.
137. Youmans, G. P., and Raleigh, G. W.: The use of mice in experimental chemotherapy of tuberculosis. III. Histopathologic assay of chemotherapeutic action. J. Infect. Dis., 1948, **82:**221–225.
138. Youmans, A. S., and Youmans, G. P.: Studies on the metabolism of *Mycobacterium tuberculosis*. II. The effect of compounds related to the Krebs tricarboxylic acid cycle on the growth of *Mycobacterium tuberculosis* var. *hominis*. J. Bact., 1953, **65:**96–99.
139. Youmans, A. S., and Youmans, G. P.: Studies on the metabolism of *Mycobacterium tuberculosis*. III. The growth of *Mycobacterium tuberculosis* var. *hominis* in the presence of various intermediates of the dissimilation of glucose to pyruvic acid. J. Bact., 1953, **65:** 100–102.
140. Youmans, G. P., and Youmans, A. S.: The relation between the size of the infecting dose of tubercle bacilli and the survival time of mice. Amer. Rev. Tuberc., 1951, **64:**534–540.

141. Youmans, G. P., and Youmans, A. S.: Studies on the metabolism of *Mycobacterium tuberculosis*. I. The effect of carbohydrates and alcohols on the growth of *Mycobacterium tuberculosis* var. *hominis*. J. Bact., 1953, **65:** 92–95.
142. Youmans, G. P., and Youmans, A. S.: Response of mice to standard infecting doses of *Mycobacterium tuberculosis* var. *hominis*. Proc. Soc. Exptl. Biol. Med., 1955, **90:**238–240.
143. Youmans, G. P., Parlet, R. C., and Youmans, A. S.: The significance of the response of mice to immunization with viable unclassified mycobacteria. Amer. Rev. Resp. Dis., 1961, **83:**903–905.

Chapter 32

MEDICAL MYCOLOGY: THE PATHOGENIC ACTINOMYCETES, MOLDS, YEASTS AND RELATED MICROORGANISMS

The discovery of the causal relation of certain of the fungi to infectious disease preceded the pioneer work of Pasteur and Koch with the pathogenic bacteria by several years, for Schoenlein studied the fungus causing favus in 1839, and in the same year Lagenbeck described the yeast-like microorganism of thrush. In spite of its earlier beginnings, medical mycology was soon overshadowed by bacteriology and has never received as much attention, though some of the fungous diseases are among the more common infections of man. This is perhaps attributable to the relatively benign nature of the common mycoses and the rarity of the more serious ones, and to the morphological basis of the differentiation of these structurally complex forms which, in a practical sense, sets them off sharply from the bacteria.

Even a brief consideration of the fungous diseases makes it clear that they fall into two well-defined groups, the superficial mycoses and the deep-seated mycoses. The superficial mycoses are by far the most common, are caused for the most part by a relatively homogeneous group of fungi, the *dermatophytes,* and include the various forms of tinea or ringworm with infection of the hair and hair follicles, the superficial infections of the intertriginous or flat areas of the glabrous skin, and the onychomycoses or fungous infections of the toe and fingernails. In general, the lesions are mild, superficial, and restricted; the infections are almost never fatal. The causative microorganisms are parasitic, and the infection is transmitted from one host to another. The deep-seated mycoses, on the other hand, are relatively rare and are heterogeneous in etiology; they include actinomycosis, aspergillosis, sporotrichosis, blastomycosis, coccidioidomycosis, and cryptococcus or torula infections. The causative microorganisms appear to be free-living parasites for the most part and are usually not transmitted from one individual to another, but the lesions they produce are severe, deep and spreading, and a fatal outcome is not uncommon.

The criterion of pathogenicity is one of the poorest that can be used in the differentiation of microorganisms, not only because it is variable and difficult to determine, but because by its use parasitic microorganisms are grouped together that are, in fact, much more closely related to certain free-living forms than they are to one another. The

superficial nature of pathogenicity as a differential characteristic is nowhere better illustrated than among the fungi, for the pathogenic forms which constitute the subject matter of medical mycology form a heterogeneous group and include some of the actinomycetes, certain molds and mold-like fungi, and a number of yeasts and yeast-like organisms. The pathogenic varieties of these fungi are an almost infinitesimally small portion of existing fungi, the vast majority being saprophytic forms found in the soil and elsewhere playing a role of primary importance in the cyclical transformations of organic matter, especially in the decomposition of hemicelluloses and lignins. A number are of great industrial importance, as in cheese manufacture and saccharification of starches. From a general biological point of view, then, the pathogenicity of certain fungi is of very minor significance; from that of the parasitized host, man, it is of considerably greater interest.[109]

The fungi are structurally complex, showing a variety of reproductive structures associated with sexual and asexual processes, in addition to vegetative nonreproductive elements. Their differentiation into genera, species, and varieties is made in large part on a morphological basis,[25, 78, 118] especially the morphology of the reproductive structures, and, in contrast to the bacteria, their physiological and immunological characteristics are usually of minor or no importance for purposes of differentiation or identification. The biochemistry of the fungi, and the molds in particular, has been extensively investigated in connection with the elucidation of decompositions occurring in nature and their application to industrial processes. Present knowledge of the respiratory mechanism of the cell and of alcoholic fermentation derives in very large part from studies of yeasts. The immunological properties of the fungi have been little studied aside from noting the immunological relation of certain of the actinomycetes to the tubercle bacilli, and the allergic phenomena associated with infection with certain of the dermatophytes and yeast-like fungi.[93]

Though a great many species of fungi have been described as pathogenic for man and animals, not all are of equal importance. Some, especially among the dermatophytes, are not legitimate species different from those already known. Furthermore, in many cases the fungus described probably had no causal relation to the pathological process from

The Fungus Infections

TYPE	CAUSATIVE ORGANISM	DISEASE
Superficial infections	Dermatophytes: *Microsporum*, *Trichophyton*, *Epidermophyton*	Ringworm of scalp and glabrous skin, suppurative folliculitis, onchomycosis
	Nocardia minutissima	Erythrasma
	Fonsecaea pedrosoi and its varieties	Chromoblastomycosis
	Candida albicans and related forms	Moniliasis of the mucous membranes and nails, sometimes generalized
	Malassezia furfur	Tinea versicolor
Deep-seated infections	*Actinomyces bovis*, *Nocardia asteroides*, *N. madurae*, etc.	Actinomycosis, including actinomycotic mycetoma
	Aspergillus fumigatus, and others	Aspergillosis, otomycosis and pulmonary mycosis
	Allescheria boydii et al.	Maduromycosis
	Sporotrichum schenckii	Sporotrichosis
	Cryptococcus neoformans	European blastomycosis, torula meningitis
	Blastomyces dermatitidis	North American blastomycosis
	Blastomyces brasiliensis	South American blastomycosis
	Coccidioides immitis	Coccidioidomycosis
	Histoplasma capsulatum	Histoplasmosis

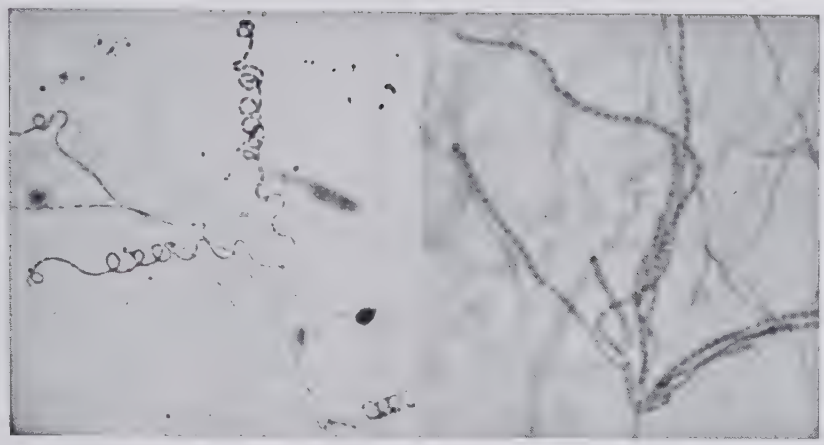


Figure 184. *Left,* Actinomycetes showing branched filaments with coiled terminal bodies which are fragmented into bacteria-like cells. *Right, Actinomyces antibioticus* showing both submerged mycelium and aerial sporulating mycelium. × 1200. (Starkey, Waksman, and Woodruff.)

which it was isolated, and in others only one or two cases of infection have ever been observed. It is the accumulation of large numbers of species of fungi and the minutiae of their morphological differentiation that give medical mycology its complexity. Here we shall be concerned only with the more important fungi known to be causally related to human disease[67]; the remainder, though large in number, are associated with only a small fraction of one per cent of fungous disease of man. These do not, in general, appear to be special pathogenic forms, but rather the diseases they produce represent infections with normally saprophytic or nonpathogenic parasitic forms. In fact, the great majority of fungi found in disease have also been found to be free living in nature, commonly as soil saprophytes.[2]

Antifungal Antibiotics.[15, 174] Classic actinomycosis, caused by *A. israeli* (see below), may be successfully treated with penicillin, but otherwise the various pathogenic fungi are not affected *in vitro* by the antibacterial antibiotics or other chemotherapeutic agents. Conversely, the antibiotics effective in the therapy of fungous infections have little or no antibacterial activity.

A group of antibiotic substances formed by various species of Streptomyces,[6] the polyene antibiotics,[34, 129] are effective against certain of the pathogenic fungi. They have the general structure $(CH{=}CH)_n$ and, of the dozen or so which have been described, nystatin[77] and amphotericin B[63] have proved useful. Nystatin is a diene-tetraene polyene and amphotericin B a heptaene. Both contain the diaminomethyldeoxypentose, mycosamine, and nystatin also contains lactone structures. Both are relatively insoluble in water or saline. Nystatin is effective in Candida infections (moniliasis) but not in the deep mycoses or dermatophyte infections. Its activity is complemented by that of amphotericin B which is effective against the deep mycoses in which the fungus is in a yeast phase in the tissues, *e.g.*, blastomycosis, cryptococcosis, histoplasmosis, coccidioidomycosis and perhaps sporotrichosis, but its usefulness tends to be limited by its toxicity.[123, 154]

Another antibiotic, griseofulvin,[142] produced by *Penicillium griseofulvum*, is an effective chemotherapeutic agent in the dermatomycoses, but not in the deep mycoses.[42] It has been found to be 7-chloro-2′,4,6-trimethoxy-6′-methylspiro [benzofuran-2(3H),1′-(2)cyclohexene]-3,4′-dione. It may be applied topically, and when it is given by mouth it is absorbed and concentrated in the keratin-containing tissues. This antibiotic was isolated in 1939 but was of no interest at that time because it has no antibacterial activity. It was used for the treat-

ment of some fungous infections of plants, but its chemotherapeutic activity in mammalian dermatophyte infections was not observed until 1958. Since then it has been increasingly widely, and successfully, used for the treatment of the dermatomycoses.

THE ACTINOMYCETES[45, 189]

The actinomycetes occupy an uncertain position with respect to the bacteria on the one hand and the fungi on the other and are regarded by many as transition forms. They grow in the form of fine straight or wavy nonseptate filaments or hyphae 0.5 to 0.8 μ in diameter which show both lateral and dichotomous branching and which may grow out from the medium to form an aerial mycelium. On solid mediums the filaments occur in tangled masses while in liquid mediums there is a tendency to centers or clumps of growth. Small oval to rod-shaped spores 1 to 2 μ long are formed by aggregation of the protoplasm in some of the hyphae, spore formation progressing from the tip. The maturation of the spore-bearing hyphae is often associated with the formation of spirals which range from open, barely perceptible coils to those which are so compressed that adjacent turns are in contact. The spirals may be dextrorse or sinistrorse, and both direction and tightness of coiling are constant within species. The spores are more resistant than the filaments and will survive 60° C. for as long as 3 hours but are less resistant than bacterial spores. The tips of other filaments may become swollen and club-shaped. Segmentation of the filaments occurs, in some species as early as 24 hours while in others it is delayed 3 weeks or more; the segmented filaments break up to form bacillary bodies 4 to 6 μ in length which are morphologically indistinguishable from many true bacteria. In most smear preparations of the pathogenic forms, the filaments are broken up and the appearance is that of ordinary bacilli. All, or practically all, of the actinomycetes are gram-positive, and some of the pathogenic forms are partially acid-fast; these last are immunologically related to the mycobacteria.

Both spores and fragments of mycelium grow in subculture. On solid mediums the growth of the aerobic forms is dry, tough, and leathery, sometimes wrinkled, adherent to and piled above the medium; in many instances it resembles that of the mycobacteria. In some cases the growth appears powdery or chalky owing to the formation of aerial mycelium. In liquid mediums growth occurs in the form of a dry, wrinkled surface film or, more often, as flakes or aggregates which adhere to the sides of the flask, especially at the surface, or sink to the bottom.

Pigment formation, with colors ranging over the entire spectrum, is common among the actinomycetes, and differentiation is usually made between pigmentation of the vegetative mycelium and the spore-bearing aerial mycelium as well as pigment diffusing into the medium. Soluble purple and brown pigments are often observed on protein-containing mediums. The actinomycetes, especially the saprophytic forms, are physiologically active, utilizing a variety of carbon and nitrogen compounds, and many are actively proteolytic. An earthy to musty odor like that of freshly turned soil is produced by many species. The optimum temperature for growth is usually 20° to 30° C., though some of the pathogenic species grow at 37° C., and thermophilic species, analogous to thermophilic bacteria, are known. The great majority of actinomycetes are aerobic, but some of the pathogenic forms are anaerobic or at least must be cultivated under reduced oxygen tension.

Differentiation of the actinomycetes

from one another is in part on a morphological and in part on a physiological basis, the latter including pigmentation and proteolytic activity. The question has been considered by Waksman and Henrici[190] who separate the two groups by making them different families, the family Actinomycetaceae being retained for the pathogenic forms and including two genera, Actinomyces for the anaerobic forms, and Nocardia for the aerobic forms. A new family, Streptomycetaceae, is created for the saprophytes.

ACTINOMYCES BOVIS

Though the term actinomycosis refers to any infection with an actinomycete, it is often thought of in connection with disease caused by *Actinomyces bovis*. This is the most clearly defined and best studied actinomycete infection, and the disease occurs chiefly in cattle and in few other animals; it is also occasionally seen in man. Although the disease was undoubtedly observed early in the nineteenth century, actinomycotic tumors being described by Leblanc in 1826 under the name of osteosarcoma, it was first recognized as a specific parasitic disease by Bollinger in 1877. At his instigation the fungus was studied by the botanist Harz who described it and named it Actinomyces or ray fungus, because of the raylike structure of its growth in the tissues, but did not cultivate it. In 1891 Wolff and Israel isolated a micro-aerophilic actinomycete from pathological material by anaerobic culture, and in the same year an aerobic actinomycete was isolated by Bostroem from similar sources and named *A. hominis* (sometimes called *A. graminis*). Bostroem's organism has been proved to have been a contaminant, however, and it is definitely established that the organism isolated by Wolff and Israel (called by some *A. israeli*) was that observed by Bollinger and Harz and the etiological agent of the disease. These early observations were extended in large part through the work of Wright and Emmons, and summarized by Erikson.[44]

In the opinion of some workers there are distinct types of this microorganism, one corresponding to the rough form that is *A. israeli* and usually responsible for human actinomycosis but occasionally found found in cattle, and the other corresponding to the smooth form that is *A. bovis*, the usual cause of actinomycosis in cattle and rarely found in the human disease. Pending more general agreement, the two will not be separated

The Pathogenic Actinomycetes and Actinomycete-like Fungi

ORGANISM	DISEASE	GEOGRAPHIC DISTRIBUTION
Actinomyces bovis (*Actinomyces israeli*)	Actinomycosis in man, "lumpy jaw" in cattle	Ubiquitous; majority of cases reported in U. S.
Nocardia madurae *Nocardia bahiensis* *Nocardia somaliensis*	Actinomycotic mycetoma in man	Tropics, including India and Egypt; few cases in U. S.
Nocardia asteroides	Deep-seated abscesses and pulmonary infection	Ubiquitous
Nocardia gypsoides *Nocardia brasiliensis*		Mexico
Nocardia keratolyticus	"Cracked heels"	India
Nocardia minutissima	Erythrasma	Ubiquitous
Nocardia farcinica	Animal parasites, do not infect man	Europe and Australia

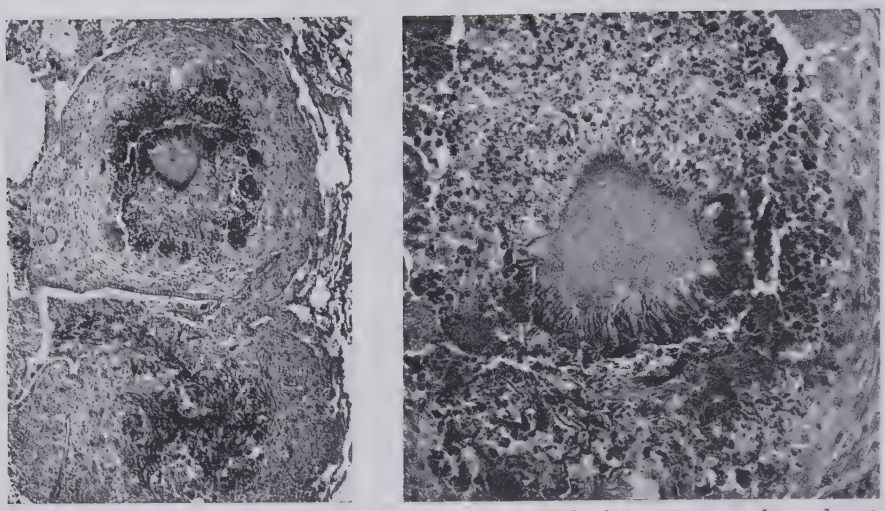


Figure 185. Actinomycotic (*Actinomyces bovis*) granule in rabbit lung. Hematoxylin and eosin. *Left*, two granules. × 80. *Right*, upper part of left field at higher magnification. × 300. Note the eosinophilic clubs at the periphery of the granule. (Rosebury, Epps, and Clark.)

here but considered as a single species, *A. bovis*, occurring as rough and smooth variants.

Morphology and Staining. Actinomycosis is essentially a suppurative process, characterized by the formation of granulation tissue, fistulation, sinus formation, and by the presence in the pus of yellow granules ("sulfur granules"), the *drüsen* of German writers. These are in fact colonies of the fungus, and when examined microscopically are seen to consist of dense rosettes of club-shaped filaments in radial arrangement. The individual rosettes are usually 30 to 40 μ in diameter but sometimes are as large as 200 μ. The minute yellow granules, visible to the naked eye, may consist of a single rosette or may be made up of several. The rosette itself is made up of three kinds of structures: a central core of branching filaments, irregularly disposed but with a general radial arrangement; refringent, club-shaped bodies at the periphery radially arranged; and spherical coccus-like bodies. The granules may be crushed and examined in fresh preparations in which the clubs may be plainly seen or a stain such as eosin may be used which colors the sheath of the clubs. The filaments are gram-positive and the stain is useful for tissue sections; hematoxylin-eosin is quite satisfactory for sections. *A. bovis* is not acid-fast.

The filaments of the central core are branched, are often curved, sometimes spirally, and are thickly interlaced in a network of mycelium. The individual filaments have a granular appearance and, particularly in older granules, segmentation and fragmentation are common, giving the filaments the appearance of chains of cocci.

The club-shaped bodies at the margin of the granule are conspicuous for their high refringency and general structureless, homogeneous appearance. They are pear-shaped swellings of the terminal ends of the filaments and arise as distinct transformations of these. In young colonies the hyaline substance of which the clubs are composed is soft and may be disolved in water, but as the age of the colony increases, the clubs become of firmer consistency. Their formation appears to be associated with the resistance of the tissues; when resistance to invasion is slight, they are absent, filaments alone being found. Clubs are, as a rule, more common in bovine than in human lesions.

The coccus-like bodies reported by various observers are probably of diverse nature. Such forms may result from the segmentation and fragmentation of filaments; in other cases they may be the ends of clubs appearing in the field of focus of the microscope. In still others they may be micrococci, secondary in-

Figure 186. *Actinomyces bovis* from cultures. Left, darkfield. × 900. *Middle* and *right*, Gram stains of rough and smooth cultures respectively. × 1200. (Rosebury, Epps and Clark.)

vaders of the suppurating actinomycotic lesion.

Morphology in culture. The colonial morphology of *A. bovis* grown anaerobically on solid mediums is sufficiently distinctive that, with experience, the colonies are readily differentiated from those of contaminating bacteria. After 4 to 6 days' incubation the colonies are often less than 1 mm. in diameter. They are usually opaque, dead white, or rarely showing a slight gray or yellow tinge, and have a pitted glistening surface and irregular margin. The growth, piled high above the surface of the medium, is similar to that of the tubercle bacillus. The colonies are tough and leathery, often coming away from the medium in one piece, and are difficult to emulsify. It has been observed that after continued cultivation the colonies tend to become smooth and less distinctive in appearance and may be much softer in consistency. In shake cultures in dextrose agar the fungus grows below a depth of perhaps 10 mm. and appears as irregularly shaped, opaque, whitish nodules which may reach a diameter of 2 to 3 mm. in a week. Stab cultures in dextrose agar give a dense grayish streak of small colonies along the lower part of the line of inoculation. In broth good growth usually occurs in the form of solid, whitish, mulberry-like granules at the bottom of the tube; the broth does not, as a rule, become cloudy.

The essential features of the rosette have been reproduced in cultures. The smaller colonies are rounded masses of branching and interlacing filaments. As the filaments become older they tend to fragment and the largest colonies are dense opaque masses of short filaments and rod forms. Clubs are not formed in the usual mediums but only in the presence of blood, serum, or ascitic fluid and even there develop inconsistently; it is generally agreed that club formation in tissue is in large part a host response. Stained smears of cultures show largely bacillary forms and a few fragments of branching filaments. The bacillary forms may be diphtheroid in appearance, stain irregularly, and some have terminal swellings; the last are quite unlike the peripheral clubs of the actinomycete granule.

Physiology. Particular interest has centered about the relationship of *A. bovis* to oxygen. In the past it has been assumed that this microorganism is an obligate anaerobe, and the identity of strains showing even scanty growth under aerobic condition has been questioned. It now seems to be established that it is micro-aerophilic with a limited oxygen tolerance, and some strains may grow sparsely in the air for one or two transfers. It is, however, best cultivated under anaerobic conditions in the presence of 5 to 10 per cent carbon dioxide. The limits in pH are similar to those of

most bacteria, and growth is best at 37° C. Growth is relatively slow even under optimum conditions, and 4 to 6 days' incubation are usually required.

There appears to be no definite information regarding the nutritive requirements of the fungus; in general it does not appear to be fastidious in that reasonably good growth occurs in meat extract mediums with added dextrose (1 per cent). Rosebury, Epps, and Clark[141] have found brain heart infusion agar most satisfactory and not improved by the addition of blood. Growth on potato is scanty, and milk is not a good culture medium. It has been a general experience that *A. bovis* cultures are difficult to maintain in the laboratory and usually die out in spite of weekly or biweekly transfer. Erikson[44] has recommended alternate transfers on different mediums for the maintenance of cultures, and Rosebury, Epps, and Clark report good results with this method. Glucose, maltose, lactose, and salicin are fermented with acid and no gas, indol and hydrogen sulfide are not produced, and nitrate is actively reduced. These biochemical reactions have no differential significance.

Antigens. By the use of the reciprocal agglutinin absorption method, and subsequently fluorescent antibody, Slack and his associates[160, 161, 162] have shown that the actinomycetes are separable into four serotypes, designated A, B, C and D. The close relationship of these microorganisms with the corynebacteria is indicated by the cross reaction of *C. acnes* with group A. The serotypes do not appear to coincide with habitat, and their relationship to immunity (see below) is not known.

Pathogenicity for Animals. The majority of actinomycotic lesions in cattle are found in or about the head; the lower jaw in particular is affected, hence the common name of "lumpy jaw" for this disease. In addition to the growth in the tongue and maxillary bones, actinomycotic lesions occur in the pharynx, lungs, skin, lymph glands, and subcutaneous tissue, especially of the head and neck, and occasionally in other organs, notably the liver. The growth of the parasite usually leads to the formation of a hard tumor which gradually increases in size and burrows into the adjacent tissues, softening and disintegrating the bony structure of the head, and at the same time forming new tissue, so that great distortion often ensues. The extension of the disease takes place by gradual invasion of the contiguous tissues, metastases being uncommon. When death occurs it is not, as a rule, due to any toxic effect but wholly to the mechanical action of the tumor in pressing upon or occluding the respiratory passages or in interfering with the taking or mastication of food. Generalized actinomycosis is rare. When it

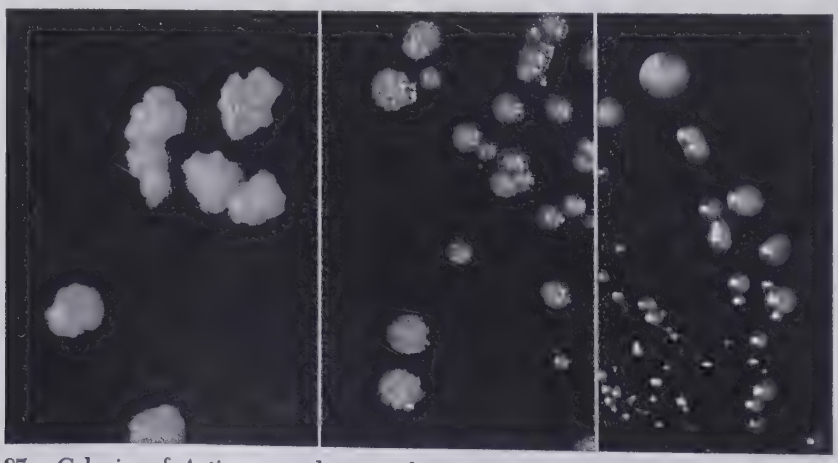


Figure 187. Colonies of *Actinomyces bovis* on brain heart infusion agar after 6 days' incubation. *Left* and *center* are rough types. × 3. The colonies on the right are the smooth type. × 6. (Rosebury, Epps, and Clark.)

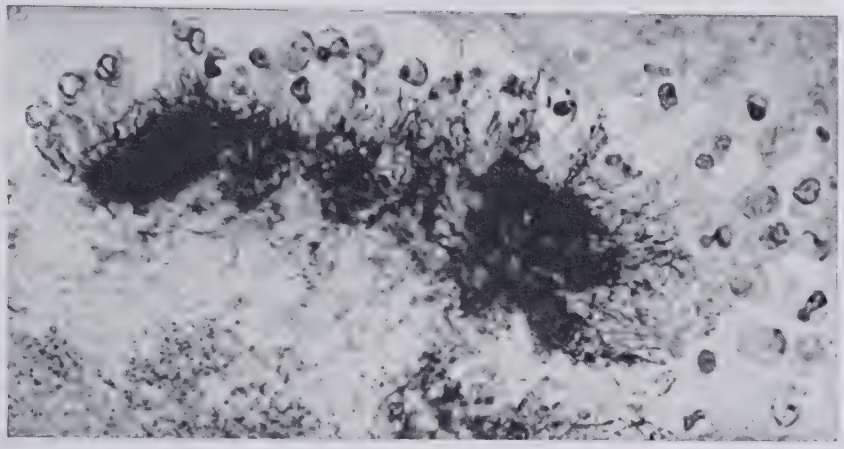


Figure 188. Pulmonary infection in man with *Actinomyces bovis*. Margin of a granule in lung tissue showing the ray-like structure. Note the bacteria designated *Actinobacillus actinomycetem-comitans* in the center of the granule. Gram stain: × 1000. (Humphreys.)

occurs the bloodstream rather than the lymph seems to be the channel by which the disease is spread. Secondary abscesses are found mainly in the liver.

The characteristic yellow granules or *drüsen* are found in the suppurating mass of the tumor; if the pus is shaken up with water in a test tube the small granules become evident and sink to the bottom of the tube. These structures are so typical that their demonstration by microscopic examination in a case of obscure suppuration is sufficient to suggest a diagnosis which must be substantiated by crushing the granules and staining for the typical forms. The new growth—granulation tissue—consists chiefly of epithelioid and spindle-shaped connective-tissue cells; small giant cells are sometimes present. To the naked eye, actinomycotic lesions in the lung and udder often resemble tuberculous nodules and at times have undoubtedly been mistaken for them; microscopic examination suffices to establish their nature.

The incidence of actinomycosis in cattle is uncertain, in part because it is readily confused with actinobacillosis; possibly something under 1 per cent is a fair estimate. Of the other domestic animals swine are most frequently affected. Horses and sheep are affected less often, and the disease seldom occurs in dogs and is rare in cats.

Experimental animals. Attempts to infect experimental animals with *A. bovis* have in general been disappointing in that only a small proportion of inoculated animals develop the disease, and the lesions are limited and benign. Henrici and his associates found that repeated inoculations are much more effective; Slack[159] was able to produce chronic progressive actinomycosis in this way in four of five rabbits. Rosebury, Epps, and Clark were less successful, and by repeated inoculation of twenty-four rabbits and sixteen guinea pigs produced infection in only five animals, progressive and fatal in two guinea pigs and one rabbit, and localized and benign in two rabbits. In the experimental disease, however, the essential features of the natural infection are observed, including the formation of tubercle-like nodules and the development of structurally typical granules with clubs.[60]

Pathogenicity for Man.[27, 72] Infection with *A. bovis* is observed in man from time to time, but it is not common. Sanford found about 700 reported cases in the United States up to 1923; a large proportion were in the upper Mississippi Valley, a distribution which probably represents recognition rather than incidence of the disease.

The disease in man differs in minor re-

spects from that in cattle. Actinomycotic infections of the bone are relatively less frequent, the disease being confined to the softer parts in most cases. There is usually a lesser production of new tissue and a more extensive softening and suppuration. The disease falls into three clinical types. About 60 per cent of the infections are *cervicofacial,* and this type is often associated with dental defects or accidents and is a chronic, localized form of the disease which is relatively benign and usually susceptible to treatment. Some 14 per cent of the cases are *thoracic* infections, and 8 to 18 per cent are *abdominal,* in which the primary lesion is often in the appendix; in these types prognosis is poor. Draining sinuses are usually found in all types, and abscesses are frequently observed in the liver at autopsy. Generalization by hematogenous spread is occasionally seen and is relatively more common in man than in cattle. Meningitis and endocarditis have been reported. The characteristic granules with clubs are usually found in the pus but may be absent occasionally in draining sinuses, especially when the infection has spread rapidly, particularly in meningitis and empyema. The disease may terminate fatally in a few weeks through secondary infection or formation of emboli, or it may drag along in a chronic form for many years; spontaneous healing has been observed.

Epidemiology. Direct transmission of *A. bovis* from one animal to another or from cattle to man has never been satisfactorily established. Many of the cases of actinomycosis reported in man are among persons who have not been engaged in agricultural pursuits and, so far as discovered, have not come in contact with any pre-existing cases in animals or man. Nor has *A. bovis* been found to exist as a saprophyte in nature. There has been some confusion on this point since Bostroem's aerobic actinomycete is a widely distributed saprophyte; the Wolff-Israel organism, however, appears to be a strict parasite and as such is widely distributed in the normal human mouth and throat. Actinomycetes make up a large part of dental plaque material, and fungi indistinguishable from *A. bovis* and capable of producing experimental actinomycosis in animals have been found in the absence of actinomycosis in carious teeth, dental and gingival scrapings, and tonsillar crypts by many workers.[140] In fact is was early predicted by Wright that *A. bovis* would be found to be a normal inhabitant of the mouth. In the light of present knowledge it seems highly probable that the infection is endogenous in origin and that the fungus, normally present but of low pathogenicity, multiplies in the favorable environment provided by necrotic tissue resulting from injury and occasionally establishes a focus of infection, possibly in association with bacteria which are eliminated as the infection progresses.

Immunity. There appears to be little effective acquired immunity to *A. bovis* infection. Recently the possibility of the development of hypersensitivity to the actinomycete cell substance has received some attention. The apparently greater efficacy of repeated small doses over a single massive inoculation in the experimental production of the disease has provided the basis for Henrici's suggestion that infection may depend on previous sensitization. This is not established as yet.

Isolation and Diagnosis. As indicated earlier, the demonstration of the typical actinomycete rosette in the tissue or pus of a specimen is sufficient to establish a diagnosis of actinomycosis in man. Actinomycosis in cattle may be confused with actinobacillosis; the two are readily differentiated by examination of a gram-stained smear for the presence of gram-positive diphtheroid-like fragments of actinomyces filaments or of gram-negative actinobacilli. As may be inferred from the foregoing discussion, animal inoculation is useless as a diagnostic method. Cultivation of the fungus is often, though not always, successful.

The procedure recommended by Wright for the isolation of *A. bovis* in culture has been widely used. Granules,

preferably from a closed lesion, are thoroughly washed in several changes of sterile water or broth and then crushed between sterile glass slides. The material is inoculated into liquid (40° C.) 1 per cent dextrose agar in test tubes filled to a depth of 6 to 8 cm. After incubation at 37° C. for 4 to 8 days, characteristic colonies develop, in greatest numbers in a narrow zone 5 to 12 mm. below the surface. These may be isolated from the shake culture in the usual way, washed in sterile water or broth if there is reason to suspect bacterial contamination, and subcultured in deep tubes of dextrose agar. Even in the absence of contamination the method is not particularly satisfactory and frequently fails.

Rosebury, Epps, and Clark[141] have shown that abundant and characteristic growth occurs on the surface of enriched agar mediums containing dextrose and incubated under anaerobic conditions in the presence of carbon dioxide. They recommend brain heart infusion containing 2 per cent agar. The granule, exudate, or other material is streaked serially on plates of the medium by means of a sterile bent glass rod and the plates incubated 4 to 6 days under anaerobic conditions in the presence of 5 per cent carbon dioxide. By this method. *A. bovis* can be isolated even in the presence of heavy bacterial contamination.

MYCETOMA

Mycetoma (fungous tumor) or Madura foot is an infection occurring with some frequency in southern India; the first cases were reported (1842 *et seq.*) from Madura, hence the name. It is a disease of tropical climates though a number of cases have been observed about the Mediterranean in North Africa, in Greece, and in Italy. It also occurs in South America and the Caribbean islands. It has been observed in temperate climates, a few cases having been reported from Europe and the United States. It was first observed in this country by Wright in Boston in 1898. The disease was first studied systematically by Vandyke Carter, and Chalmers and Archibald proposed the system of classification of the mycetomas which is generally used.

The disease usually affects the foot; occasionally the hand is attacked, and other parts of the body more rarely. It commonly occurs in those habitually going barefoot, and the fungus presumably enters the body through some injury or abrasion. There is some evidence that it may remain latent for some time, even a period of years, and become active following injury. The part first involved, usually the sole of the foot, shows a small subcutaneous swelling which slowly enlarges and softens to become phlegmonous. It ruptures to the surface, sinus tracts form, and the process burrows into the deeper tissues, producing swelling and distortion of the foot; the bones may or may not be extensively involved. Numerous small eminences are found on the surface, each the orifice of a sinus. The discharge is a viscid, slightly purulent, often foul-smelling fluid containing minute granular particles up to 1 mm. in diameter. The presence of these grains separates the mycetomas from pseudomycetomatous conditions observed in yaws, sporotrichosis, etc. The disease is strictly local, secondary abscesses seldom if ever occur, and the internal organs are probably never affected. Iodide and antibiotic therapy is reported to be effective in the actinomycete infections, but with extreme tissue destruction or other etiological agents, surgical removal of the infected tissues, usually involving amputation, is necessary and curative.

The most obvious clinical difference in the mycetomas is the color of the grains present in the discharge. These may be white, yellow, or black, and red grains have been observed. These grains, or colonies, are composed of mycelial filaments of the fungus; clubs are often present in the actinomycete grains, and chlamydospores in those of other fungi; the color is due to the formation of pigment by the fungus. Differentiation into

Figure 189. *Nocardia asteroides.* Smear from a pure culture, Gram stain; × 1050.

white or yellow grained types on the one hand, and black on the other, is clinically attractive but unsound since grains of the same color may be produced by widely different fungi, and in one instance both black and white grained types have been produced experimentally with the same strain of microorganism. It is customary, therefore, to divide the mycetomas into two etiological types:

(1) actinomycosis—mycetoma of actinomyocete etiology.

(2) maduromycosis—mycetoma caused by molds and mold-like organisms.

It would seem that since none of the reported actinomycotic mycetomas in which the fungus was identified showed black grains, and black grains are associated with maduromycosis, the white or yellow grained type may be either actinomycosis or maduromycosis while the black grained type is probably maduromycosis. There is in general a greater tendency to bone involvement in the actinomycete infections. Otherwise there appear to be no clinical differences between the two types.

Actinomycotic Mycetoma. A number of species of actinomycetes have been described in mycetoma. Of these two appear to be the most important, the others having been observed only occasionally. *Nocardia madurae* is widely distributed and has been found in Tunis, Egypt, Algiers, and Greece, in Senegal, Somaliland, and Abyssinia, in India and in South America and the Caribbean. *N. asteroides* was found in mycetoma in the Philippines and has since been observed in South America and in Europe. Of the others *N. bahiensis* has been found several times in Khartoum and *N. somaliensis* in Africa.

Maduromycosis. The etiology of maduromycosis is much more heterogeneous, the causative organisms including molds and moldlike fungi. Of the molds, species of Aspergillus and Penicillium have occasionally been found in maduromycosis. The other etiological agents include organisms of the genus Madurella, secreting a black pigment and producing a black grain mycetoma; there are eight species of which the first to be described, *Madurella mycetomi,* is the most common. Closely related forms producing no pigment and causing white grain mycetoma are three species of the genus Indiella; these are not common. One of the sporotrichia, Glenospora (Aleurisma), is responsible for some of the black grain and white grain mycetomas, and certain species of Monosporium (Scedosporium) cause both black and white grain mycetomas, and Allescheria species are commonly found. Experimental infections are extremely difficult to produce because of the low virulence of these fungi for experimental animals.[119]

Diagnosis. As indicated above, the mycetomas are characterized by the presence of granules in the discharge.

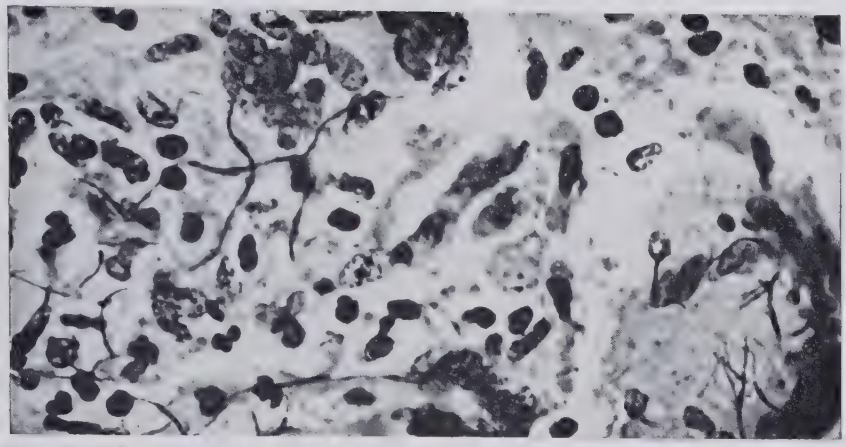


Figure 190. Bronchopulmonary actinomycosis in man resulting from infection with the non-ray type (aerobic) of actinomyces. Note the branching mycelium in the giant cells. Gram stains. × 860. (Humphreys.)

The grains may be very hard and are usually treated with strong (20 per cent) sodium hydroxide to dissolve pigment and debris and examined in a wet, unstained condition for the presence of tangled mycelial filaments. Clubs may be observed in the actinomycete infections, and thallospores, heavy-walled homogeneous structures, are found in Madurella and other infections. For differentiation and identification of the fungus, culture is necessary, on enriched dextrose agar in the case of actinomycetes and on Sabouraud's medium in the case of others.

OTHER NOCARDIA INFECTIONS

Cases of various types of infection with actinomycetes are found more often than is generally realized. *N. asteroides,* originally found in a brain abscess by Eppinger in 1890, has been observed in a variety of pathological processes including pulmonary affections sometimes called "pseudotuberculosis." Its occasional occurrence in mycetoma has been noted above. Both this organism and *N. gypsoides,* another strain of *N. asteroides,* isolated from sputum in pulmonary disease,[83, 191, 192] are acid-fast. These forms are immunologically related to the mycobacteria. A number of acid-fast actinomycetes have been isolated from similar affections and given various names. Infection occurs first in the peribronchial nodes, followed by the development of a caseous bronchopneumonia with central softening and cavitation. The disease is of about 6 months' duration and may be confused with tuberculosis; in acid-fast stains of sputum smears only the bacillary forms of the actinomycete are present and cannot be distinguished from tubercle bacilli. It may be readily differentiated by culture and guinea pig inoculation.

A disease of the foot occurring in India, cracked heels or *keratodermia plantare sulcatum,* known locally as *chaluni, haja,* or *panki,* has been reported to be caused by an actinomycete, *Actinomyces (Nocardia?) keratolyticus,* but this has not been confirmed. The fungus appears to have a lytic action on the horny layer of the epidermis, and the disease is characterized by solution of the thick horny skin of the plantar surface in grooves. The skin cracks along these grooves to form deep fissures on the heel and in the thick sodden skin between the toes which extend through the corium to the subcutaneous tissues. Secondary infection, especially with streptococci, is common.

The disease *ulcus interdigitale* may be associated with the same organism. Deep fissures develop between the toes, and large chronic ulcers appear which show little discharge and are very painful. The lesions may extend deeply into

the interdigital cleft, and secondary infection may necessitate amputation of the toe. The ulcers may also develop in the sole of the foot in the region of the heel and great toe. Infection with *A. keratolyticus* occurs during the monsoon season and appears to be contracted through walking bare-foot on damp soil contaminated with manure.

Erythrasma, a superficial mycosis resembling tinea versicolor, is caused by *N. minutissima.* The initial lesion is a small scaly macule which slowly enlarges to produce a yellowish to reddish brown scaly patch with reddened borders. The disease is most commonly observed in the axillae and groins, and the intergluteal cleft or other intertriginous areas are less frequently involved. It is not found on free nonintertriginous surfaces.

Other actinomycete infections take a variety of forms. Abscesses in various parts of the body, including the brain, are observed from time to time, and a number of cases of corneal infection and conjunctivitis have been reported.

Actinomycotic infections of domestic animals are occasionally observed. One of the first to be reported was a farcy-like disease of cattle caused by *N. farcinicus.* Other infections are seen in sheep and goats in a variety of clinical forms. A skin disease of sheep of some economic importance because of the copius exudate which prevents shearing and makes the wool unmarketable, and due to *A. dermatonomus,* has been reported from Australia.

When actinomycetes are isolated from some pathological process and studied, it is often found that the fungus does not conform precisely to previously described species, and a new species is created. Many species have been described, therefore, of which a large proportion are of doubtful importance either because of the rarity of their occurrence, as having been observed only once, or because there was some doubt as to the etiological relation of the fungus to the disease. Cultural characteristics, when carefully determined, allow the separation of Nocardia from streptomyces-like forms, and differentiation into species.[144]

THE MOLDS AND MOLD-LIKE FUNGI

The fungi proper, or Eumycetes, are quite distinct from the true bacteria, being closely related to the higher algae but differing from them in that they lack chlorophyll. Phylogenetically, their differentiation may have arisen through a degenerative loss of chlorophyll and adaptation to a chemosynthetic type of metabolism. Some of the fungi are unicellular, others are multicellular, and still others are sometimes unicellular and sometimes multicellular, that is to say, dimorphic; some of the parasitic fungi are unicellular in the tissues and multicellular in culture. For present purposes we shall consider the multicellular forms here under the head of molds and mold-like fungi, and the unicellular forms such as yeasts and yeast-like fungi in the following section, though the occurrence of intermediate forms and taxonomic considerations makes such a differentiation inconsistent.

Hyphae and Mycelium. The multicellular fungi are made up in large part of cells attached end to end to form filaments or *hyphae.* These are considerably larger in diameter than the actinomycete filaments described earlier, and the latter are perhaps to be regarded as a rudimentary type of hyphae. In some instances certain of the hyphae may be coiled to form helices. The mass of branching, intertwining, and sometimes anastomosing hyphae making up a colony of the fungus is the *mycelium.* The entire mass is also sometimes called the *thallus.*

Two main structural types of my-

celium may be distinguished. In one of these the cells making up the hyphae are not separated by cross-walls or septa, making possible a characteristic flowing of protoplasm through the multinucleate structure. Such a structure is said to be *nonseptate* or *coenocytic.* Certain of the algae are of similar structure, and the fungi thus characterized are called *phycomycetes* or algae-like fungi. In the majority of fungi, however, the hyphae are *septate,* each cell separated from the other by cross-walls. The individual cells contain a single nucleus, as in the *ascomycetes* or, sometimes two as in certain of the *basidiomycetes.* Thus these three main classes of fungi may be distinguished in part on the basis of the structure of the mature, nonsporulating hyphae, though this is not one of the main characteristics upon which their differentiation is made.

The mycelium is further differentiated into two general types which differ in function. One of these, the *vegetative mycelium,* consists of masses of hyphae within the colony, adjacent to and growing into the substrate, and is concerned with the assimilation of food materials. Fragments of such mycelium will reproduce if transferred. The other type, or *reproductive mycelium,* usually extends into the air to form an *aerial mycelium* and gives rise to reproductive bodies or spores. The mode of spore formation and the structure of the spore and spore-bearing elements are the characteristics by which the fungi are differentiated, classified, and identified.

In addition to sporulation and the vegetative growth of hyphae, many species of fungi reproduce by the separation of cells, known as *oidia,* from any part of the mycelium. These vegetative reproductive forms may give rise to new mycelium or reproduce themselves by budding like the yeasts, according to the environment in which they are placed.

Spore Formation. Two kinds of spores are to be distinguished, the *sexual spores* which are produced by the fusion of two cells which may or may not differ morphologically and which are referred to as plus and minus rather than male and female, and the *asexual spores* which arise by differentiation of the cells of the spore-bearing hyphae without fusion. Fungi for which both sexual and asexual types of spore formation are known are "perfect fungi" and are further differentiated on the basis of differences in asexual spore formation and other morphological characteristics. This group includes the phycomycetes, the ascomycetes, and the basidiomycetes.

Sexual spore formation. The most common of the phycomycetes are species of Mucor and Rhizopus. These molds produce a sexual spore known as a *zygospore* by the fusion of neighboring filaments of the same or different plants. The ascomycetes, which include a number of genera as well as certain strains of Penicillium and of Aspergillus, and also the yeasts, form sexual spores known as *ascospores* because they are contained in an *ascus* or sac. Their formation is simplest in the yeasts in which two contiguous cells fuse by means of minute tubelike processes, the nuclei unite, and the resulting single nucleus divides three times to give eight daughter nuclei. Reserve material accumulates about each nucleus, a spore wall is formed, and the cell containing this is the ascus. In most of the ascomycetes the process is somewhat more complex, and the cells which fuse may be distinguishable and are termed the *oögonium* and *antheridium.* The cell resulting from fusion gives rise to new hyphae, the next to last cell is binucleate, and these nuclei fuse and then divide to form the ascospores, *i.e.,* two nuclear fusions are involved in the entire process. The basidiospores are also formed by a complex process including nuclear fusion; this group is made up of the mushrooms, puffballs, and other fleshy fungi, none of which is pathogenic.

Fungi Imperfecti. A large group of fungi, which includes practically all of the pathogenic forms, is made up of those for which a sexual phase has not been demonstrated, and only asexual

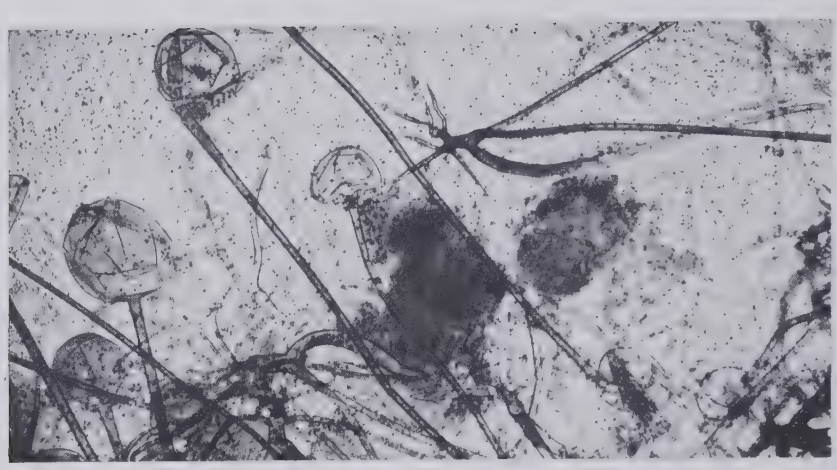


Figure 191. *Rhizopus sp.* Mounted in Amann's medium. Note the single-celled (non-septate) mycelium and ruptured, empty sporangia. The small oval bodies are free spores. The root-like structure at the base of the hyphae is the "hold-fast" by which the mold is attached to nutrient medium. × 80.

spores are produced. They are known as the "imperfect fungi" or Fungi Imperfecti and are sometimes called *hyphomycetes*; they make up the fourth group of eumycetes. The group is necessarily only provisional, and various species of fungi are removed from it from time to time as their sexual phases are discovered. It is also heterogeneous and includes species which are much more closely related to those of other groups than to one another; for example, certain species of Aspergillus are classed with the ascomycetes while others, whose sexual phase is as yet unknown, are placed with the Fungi Imperfecti.

Differential Characteristics of Genera and Species. While sexual spore formation is the basis for primary differentiation of the eumycetes, genera and species of both the "perfect fungi" and the Fungi Imperfecti are distinguished by other morphological characteristics. These, however, assume primary practical importance, especially for the pathogenic forms. They may be summarized briefly:

(1) Reproductive structures. The characteristics of asexual spore formation:

(a) In the more primitive molds, the phycomycetes, the asexual spores are characteristically produced with a capsule or *sporangium.* For example, in *Rhizopus nigricans,* the common white cottony mold found on damp bread, horse dung, etc., erect unbranched hyphae arise from the single-celled mycelium and near the apex of each a septum forms. The tip then swells into the globular sporangium, within which numerous oval spores develop; the wall of the ripe sporangium ruptures easily, and the spores are discharged by the swelling of the gelatinous mass in which they are embedded.

(b) In the higher molds the asexual spores are not enclosed in a capsule, but are produced free by segmentation of the tips of the hyphae. Such spores are known as *conidia,* and the spore-bearing hyphae as *conidiophores.* Various types of structures are developed. For example:

(i) In Aspergillus the unbranched conidiophore arises from an enlarged cell of the vegetative mycelium, designated the foot cell, and terminates in a swollen tip, the vesicle. A number of little tenpin-shaped stalks, the *sterigmata* or *phialides,* are given off from the vesicle, and the chains of conidia abstricted from these.

(ii) The common blue-green mold, Penicillium, has a similar structure; the terminal portion of certain hyphae breaks up into finger-like verticillate branches and, on the tips of these, sterigmata abstrict chains of conidia.

(iii) In Hormodendrum the formation of conidia differs from that of many other molds. Instead of successive conidia being formed by repeated constriction of the tip of the sterigmata to form a chain in which the terminal spore is the oldest, the conidiophore forms but one spore which buds to form a second, the second forms a third, etc. Furthermore, a spore may develop more than one bud simultaneously, thus producing branching chains of conidia. It may be noted that differentiation is made among these or-

ganisms on the basis of formation of phialides.

(iv) *Aleuriospores* resemble conidia in a general way in size and shape but differ in that they are formed on short lateral branches of hyphae, and sometimes directly on the hypha itself, rather than on the tips of specialized condiophores, and are not set free when mature but are liberated only when the mycelium that forms them disintegrates. When the lateral branches are very short with many spores and/or the aleuriospores are directly attached to the hyphae in considerable numbers, the structures are sometimes referred to as *hyphae sporiferae, thyrsi sporiferae,* or simply as *thyrsi.* Aleuriospores are perhaps to be regarded as lateral chlamydospores (see below) and are characteristic of the ringworm fungi in particular.

(v) *Spindle-spores* or *fuseaux* are large, thick walled structures, septate, and composed of several cells, which are formed by the aerial mycelium. Fuseaux are formed in large numbers by some species of fungi and only rarely by others and assume some differential significance, especially among the dermatophytes. Some workers regard shape, blunt or pointed ends, and other morphological characters of these bodies as valid differential characteristics.

(vi) Fragmentation of the mycelium results in the production of bodies termed *arthrospores.* These are often produced by the dermatophytes in the lesions in the skin and hair but are only rarely observed in culture. They are not true spores and are perhaps best regarded as oidia in structure and function though this point is seldom made.

(c) Large, thick-walled structures formed within the vegetative mycelium, sometimes within hyphae, at others laterally on short stalks, are designated *chlamydospores.* These structures are often irregular in form, and some are composed of several cells and approach a spindle form. In such cases they resemble fuseaux and, in fact, in much of the literature are referred to as fuseaux.

(2) Vegetative or mycelial structures. A number of structures are formed by the vegetative mycelium which have no reproductive significance but are of considerable value in the differentiation of the pathogenic fungi.

(a) *Spirals* or coiled hyphae similar to the coiled filaments of actinomycetes described in the preceding section, are observed in a number of the pathogenic mold-like fungi.

(b) *Nodular organs* are formed by some species. These are enlargements in the mycelium which consist of closely twisted hyphae, either side branches twining about the parent stem or different hyphae twisted together, and the resulting structure has a nobby, nodular appearance.

(c) *Racquet mycelium* or *mycelium en raquette* is a term applied to certain hyphae, usually larger than the others, that show a regular enlargement of one end of each segment, large and small ends being in apposition.

(d) The term *pectinate bodies* is applied to unilateral short irregular projections from the hyphae, giving a "broken comb" appearance.

(e) *Favic chandelier* is the name applied to the structure formed by the occurrence of numerous short multiple hyphal branches having the appearance of a reindeer horn. This structure is not common, occurring only in the favus fungus, *Trichophyton schoenleinii.*

(3) Colonial morphology. The morphology of colonies on Sabouraud's "differential" medium has assumed greater importance for the dermatophytes than for the other pathogenic fungi. In

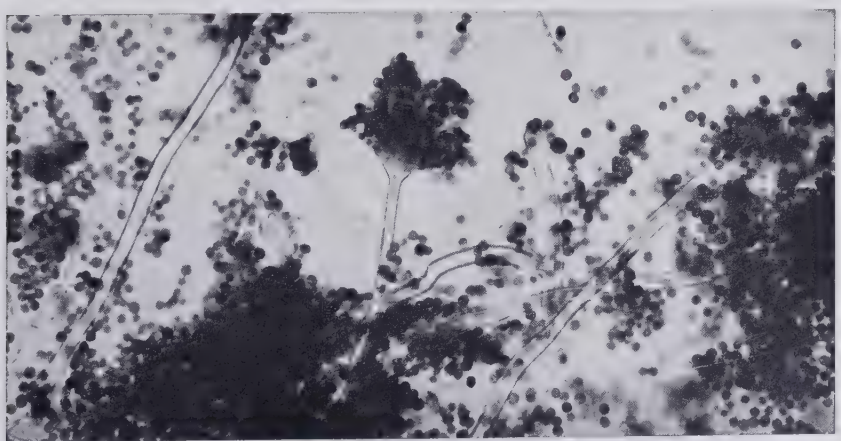


Figure 192. *Aspergillus sp.* mounted in Amann's medium. Note the fully developed sterigmata and the chains of conidia abstricted from the tips. The large dark masses are made up of free conidia. × 370.

fact, in this group primary differentiation is made on this basis and colonial morphology often serves to identify a species.

Microscopic Examination. The methods used in the microscopic examination of the fungi vary somewhat according to the nature of the material and the purpose of the examination. In general the staining methods so useful in the study of the bacteria are not applicable; the fungi are all gram-positive and may be found in gram-stained smears but their morphology is obscured. Wet, unstained, or lightly stained preparations are most informative.

Specimens. Open or draining lesions are almost always so heavily contaminated by secondary bacterial invasion that fungi are very difficult to find; in material from surgically opened lesions they are usually demonstrable though not so numerous as bacteria in corresponding bacterial infections. In the dermatomycoses secondary bacterial infection ordinarily does not interfere with the microscopic demonstration of the fungous elements. The dermatophytes live in keratin material exclusively, and specimens should be taken from scrapings of horny layers, tops of vesicles, scrapings from nail plate, and hair.

The material should be mounted in strong (10 to 20 per cent) hot sodium hydroxide, which dissolves or makes translucent the tissue elements but ordinarily affects the fungi more slowly, and examined as a wet unstained preparation. Antiformin or lactophenol may be used instead of sodium hydroxide. Care must be taken to distinguish between spores and fat globules and between mycelium and fibrin strands; a mycelial-like structure ("mosaic fungus") may be formed in some preparations, presumably from cholesterol. Fungi may be demonstrated in tissue sections, as the walls of abscesses and granulomatous tissue, by the Unna-Pappenheim (methyl green–pyronine) stain.

Cultures. A bit of growth is removed from the colony, teased apart in a drop of water, and examined as a wet preparation. While the various structures may be seen, the arrangement of the elements is seriously disturbed. Slide cultures show the structure and arrangement of the growth and may be made into permanent mounts.

Cultivation. Most of the fungi grow very rapidly, but the pathogenic forms usually grow relatively slowly and 2 or 3 weeks' incubation may be necessary. Their morphology is markedly affected by the type of medium on which they are grown. In general they show no unusual nutritive requirements and grow readily on all the usual bacteriological mediums, especially if a sugar is added. Many molds tolerate a high acidity and may be cultivated on tartaric acid-dextrose

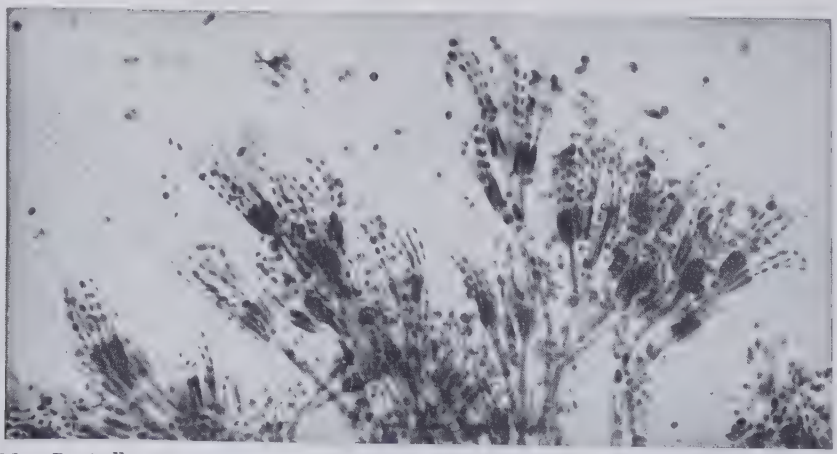


Figure 193. *Penicillium sp.* mounted in Amann's medium. Note the characteristic finger-like verticillate branches and the terminal chains of conidia. × 440.

nutrient agar on which bacterial growth is inhibited. Most grow well on modified Czapek-Dox medium (a glucose-nitrate synthetic medium) which is reproducible and has been widely used as a "standard" agar for descriptive purposes. Sabouraud's medium, a peptone-maltose agar, is perhaps the most widely used medium in medical mycology for the isolation and maintenance of cultures, especially the dermatophytes.

ASPERGILLUS (ASPERGILLOSIS)

The blue-green mold commonly observed on damp bread and similar materials is usually an aspergillus, often called *Aspergillus glaucus,* though this name has been so indiscriminately applied that it now has little or no precise significance. So far as is known, these bread molds are not pathogenic. A similar mold, however, also producing a blue-green pigment and known as *A. fumigatus,* is highly pathogenic for birds and also occasionally for man.

Pathogenicity for Animals. Aspergillosis of domesticated birds, pigeons, ducks, and chickens is not uncommon and at times assumes economic importance. Three types of infection occur, infection of the air sacs, and both nodular and pneumonic forms of lung infection. Infection of the air sacs takes the form of a superficial infection of the epithelial lining, which becomes thickened and covered with a mat of green, sporulating mycelium. In the nodular form of the disease tubercle-like masses of infiltrated tissue, necrotic in the center, are formed. A diffuse infiltration develops in the pneumonic form and the lung tissue is consolidated and grayish white in color. The pneumonic disease sometimes assumes epidemic form in chicks and is known as "brooder pneumonia." The source of infection is usually moldy grain or straw. The fungus may also invade the egg during incubation with infection of the embryo. Cattle, sheep, and especially horses develop aspergillosis though less commonly than birds; the lesions are pulmonary and may be either nodular or pneumonic.

Strains vary widely in their pathogenicity when tested by inoculation of experimental animals. In general those isolated from infections are highly virulent, while those found in air and elsewhere are of low virulence. With virulent strains a fulminating, rapidly fatal pneumonia may be produced in pigeons by inhalation of spores, while feeding grain overgrown with the mold often produces an infection of the air sacs. Intravenous inoculation of pigeons results in an acute infection with multiple miliary abscesses, especially in the lungs, if the dose is not too large. Rabbits inoculated intravenously with spore suspensions of virulent strains usually die in 3 to 5 days with multiple abscess formation, notably in the kidney, while subcutaneous or intraperitoneal inoculation produces localized lesions which may or may not be

Pathogenic Molds and Mold-like Fungi

ORGANISM	DISEASE	GEOGRAPHICAL DISTRIBUTION
Aspergillus fumigatus	Aspergillosis—pulmonary in birds, otomycosis and pulmonary infection in man	Europe and U. S.; otomycosis most prevalent in India, probably ubiquitous
Fonsecaea pedrosoi	Chromoblastomycosis	Ubiquitous with the possible exception of Europe; especially South America and Puerto Rico
Sporotrichum schenckii	Sporotrichosis	Europe and U. S., probably ubiquitous in temperate climates
Microsporum species *Trichophyton* species *Epidermophyton* species	Dermatophytosis in man, some species natural parasites of animals	In general ubiquitous, variable from species to species

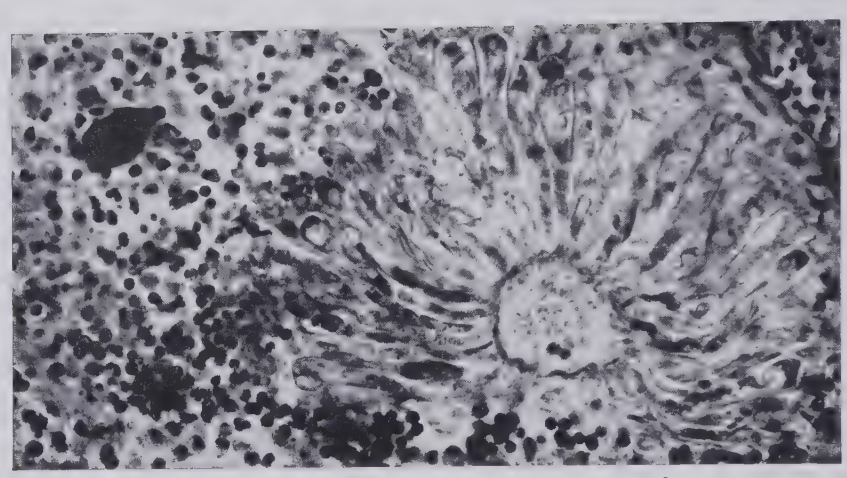


Figure 194. Pulmonary aspergillosis in man showing a fruiting head and spores in a cavity in the lung tissue. The spores are slightly smaller than red cells and show a crenated edge when viewed from the end and a banded appearance from the side. Iron hematoxylin; × 650. (Humphreys.)

fatal. Henrici[79] has shown that *A. fumigatus* produces an endotoxin.

Pathogenicity for Man. Human cases of aspergillosis are most frequently infections of the external ear (otomycosis). The disease varies from a plugging of the external meatus with a mass of mycelium to ulceration of the walls and even penetration of the middle ear; the mild cases are the most numerous. This type of aspergillosis is said to be relatively common in India, less so elsewhere though a considerable proportion (1 per cent) of ear infections in Germany have been reported to be aspergillosis. While *A. fumigatus* is the most common invader, *A. nidulans, A. flavus,* and *A. niger* are sometimes found.

Pulmonary infection is relatively rare in man and occurs in two forms, the one an intracavitary fungus mass or aspergilloma, and the other bronchopneumonic aspergillosis.[121] Infection may be secondary, particularly to tuberculosis. It is of some interest that the primary infection has been classed as an occupational disease with respect to compensation.[21] The disease is clinically similar to and may be mistaken for tuberculosis; there is extensive cavitation, and it advances rapidly. The mycelium may be found in the sputum, and the fungus is readily isolated by culture but other etiological agents must be excluded. Most of the reported cases of lung infection are among bird-fanciers, especially those having to do with the care of pigeons; there is convincing evidence that transmission of the disease from pigeons to man can take place.

Very rarely aspergillosis may take other forms in man. The occurrence of Aspergillus in maduromycosis has been noted earlier, and a case of chronic suppuration with discharge of grains has been reported. Rarely it may assume an acute disseminated form, including involvement of the central nervous system.[68]

MUCORMYCOSIS

Mucormycosis has been a very rare infection, occurring largely as a mycotic complication of chronic debilitating disease, most often uncontrolled diabetes but also amebic colitis and kwashiorkor.[111] With the introduction into general use of therapeutic agents such as antibiotics, corticosteroids and antileukemic drugs, the infection has been found more often, so much so that it is considered to be a "new disease."[5]

The fungi, usually species of Rhizopus, are apparently of very low virulence and are able to invade the tissues only when natural resistance is markedly reduced. Infection usually begins in the upper

respiratory tract, most often the nose, where the spores germinate and the mycelial growth invades the mucous membranes and extends into the adjacent sinuses and orbital cavity. Primary infection may also occur in the lung where growth on the bronchial mucosa penetrates the wall to the hilar tissue, or a lobar pneumonia may result. The fungus appears to have a special affinity for arteries with penetration into the lumen to produce thrombosis and infarctation, and may reach the central nervous system via the ophthalmic and internal carotid arteries to produce a meningoencephalitis.

FONSECAEA PEDROSOI (CHROMOBLASTOMYCOSIS)

Chromoblastomycosis was discovered by Pedroso in 1911, but his observations were not reported until 1920, the first case in the literature being reported from Boston by Medlar in 1915. The geographical distribution is wide, however, with the greatest number of cases found in Puerto Rico and Brazil, and it is quite common in Costa Rica, while others have been observed in other parts of South America, Africa, and the Far East. Present knowledge of the disease has been summarized by Carrión.[17]

The causative fungus is closely related to the Hormodendrum-Cladosporium group and was named *Hormodendrum pedrosoi* by Brumpt. Three types of sporulation are observed, the predominant type differing from one strain to another, and this has led to the splitting of strains into several genera with considerable confusion resulting. The new genus Fonsecaea was created by Negroni in 1936 and has gained some general acceptance, and the organism is, therefore, also commonly known as *Fonsecaea pedrosoi.* Since the Hormodendrum type of sporulation is not an outstanding characteristic, Carrión has proposed that the name *F. pedrosoi* be accepted as the only species and that four varieties, *typicus, cladosporioides, phialophorica,* and *communis,* be recognized on the basis of predominant type of sporulation. Mycelium is not formed in the tissues, and the parasitic phase of the fungus is a brown sclerotic cell which divides by septation.

The disease is an infectious granuloma of the skin and subcutaneous tissues, occurring usually, though not always, on the feet and legs. It ordinarily begins with a small warty growth on the foot and extends upward through the development of satellite lesions. It almost always remains localized, metastases are very rare, and there are no constitutional symptoms. The disease develops very slowly; usually the case is of 10 to 15 years' duration at the time of examination, and some are known to have persisted for as long as 40 years. In advanced cases there is some elephantiasis of the affected limb, and great numbers of lesions. These vary somewhat and are of four general types: hard, elevated, pigmented nodules; large, cauliflower-like, prominent tumors; moderately elevated, dull red, scaly patches; and discrete or diffuse verrucous hyperkeratotic growths. The lesions are readily traumatized, and the disease may be complicated by secondary bacterial infection and ulceration. The sclerotic cells appear as spherical bodies, perhaps 12 μ in diameter, with a thick membrane and granular protoplasm, and often show internal septation. They may be observed in biopsy specimens either within giant cells or free in the tissues, and are demonstrable in the epithelial debris obtained by scraping the lesions. The fungus may be cultured from such scrapings or from infected tissue. Chemotherapy is not satisfactory, iodide in large doses giving local improvement but not cure; excision of the early papillomatous lesion is recommended.[14]

There appears to be no evidence of spread of the infection from one person to another. Since the disease tends to occur on the legs and feet of barefoot, outdoor laborers in the tropics, it seems probable that the fungus is a saprophyte normally present in soil or decomposing organic matter which, when introduced into the skin by a splinter or thorn or

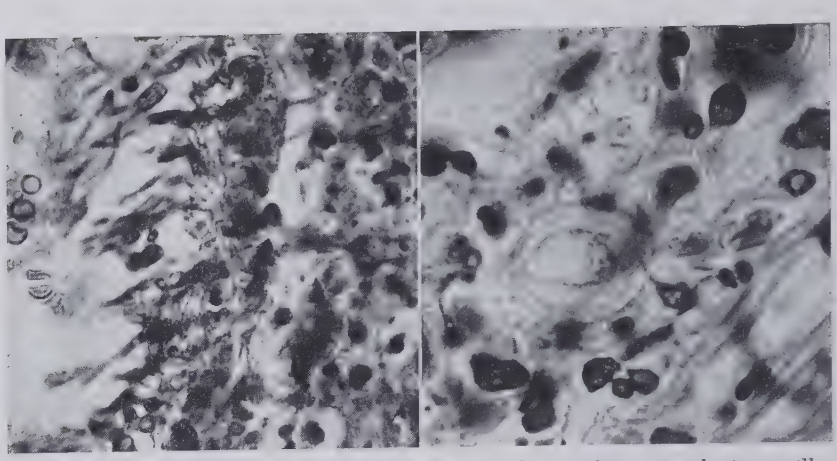


Figure 193. Primary mycotic bronchitis in man resulting from infection with *Aspergillus sp. Left,* mycelium invading the wall of the bronchus. × 950. *Right,* a segment of swollen mycelium in the deeper tissues under higher magnification. × 1550. Hematoxylin and eosin. (Humphreys.)

through some minor abrasion, may at times assume pathogenicity.

SPOROTRICHUM SCHENCKII (SPOROTRICHOSIS)

Sporotrichosis was first recognized by Schenck in this country in 1898 and a few years later in France by Beurmann and Ramond. It has since been found all over the world, though the majority of reported cases are from the United States, especially from the Mississippi and Missouri Valleys, and from France. It occurs as an occupational disease in pottery workers in Cuba, and the source of infection is presumably the packing material.[65] The disease, together with blastomycosis, is perhaps the most common and most important of the serious mycoses.

Fungi of the genus Sporotrichum are characterized by the production of pear-shaped conidia directly from the mycelium which arise both laterally and at the tips of hyphae. The characteristic arrangement is not apparent in smears from cultures, and the spores are usually found free, but is readily demonstrable in slide cultures. The hyphae are considerably more slender, 2 μ in diameter, than those of most molds. The character of the growth on agar is different from that of other molds; the colony is first soft and creamy in consistency, becoming more firm as the culture grows older, and there is no cottony mass of aerial mycelium. The growth also becomes darker with age, being at first a light tan which deepens to a dark brown or even almost black. When cultured on Francis' cystine agar at 37° C., it grows as a budding yeast and is quite as "yeast-like" as Blastomyces or Histoplasma. Mycelium is not formed in the tissues, and the parasitic phase of the fungus is a cigar-shaped body resembling an elongated yeast cell, 1 to 3 μ in breadth and 2 to 10 μ in length. These bodies are found within the leucocytes and apparently reproduce by budding; they are rarely observed in human infections. They are not spores.

The organism described by Schenck was named *Sporotrichum schenckii.* That isolated in France was supposed to be a different species and came to be known as *S. beurmanni.* The differences between the two are pigmentation, *S. schenckii* being the lighter, the formation of fewer lateral spores by *S. schenckii,* and the fermentation of sucrose but not lactose by *S. beurmanni* and the reverse by *S. schenckii.* There also appear to be some differences in the clinical type of disease produced. These differences are not constant, however, and are subject to environmental modification, and it is apparent that the two are but a single species, *S. schenckii.*

Figure 196. *Sporotrichum schenckii* in culture. Spores and hyphae. Unstained preparation; × 450 (Rothman.)

The name *S. beurmanni* continues to persist in the literature, however, giving the impression of two recognized species. The fungus infecting horses, *S. equi,* is also identical with *S. schenckii.*

Pathogenicity for Man.[127] The most common form of sporotrichosis in this country is cutaneous; the primary lesion, appearing at the site of some minor injury, often on a finger, fails to heal, ulcerates and is followed by the appearance of a series of subcutaneous abscesses along the course of the regional lymphatics. The subcutaneous lymph vessels can often be traced as reddened lines. In the series of cases reported by Gonzales Bonavides,[65] the disease occurred on the hand in 37.5 per cent, on the forearm in 25 per cent, on the arm in 6 per cent and on the leg in 9 per cent, with lymphangitic extension in 37.5 per cent. Infection seldom extends beyond the regional lymph nodes, and cases of generalized hematogenous infection are rare in this country though apparently more common in France. Metastatic lesions may appear in the liver or lungs and are most common in the testicles. The firm nodules in the skin are suggestive of syphilitic gummata, and probably some cases of sporotrichosis have been so diagnosed. Ocular sporotrichosis also occurs but is quite uncommon.[67] Pulmonary involvement has occurred but is very rare.[153]

Epidemiology. Direct transmission of the infection from man to man has been observed but is very rare. A certain number of human infections have also been contracted, either directly through bites or indirectly by contact, from naturally infected horses and rats. In the great majority of cases, however, the fungus is introduced into the tissues from plants through some abrasion. Thus in fourteen of a series of eighteen cases the infection followed wounds by barberry thorns. The disease has been observed in florists and the fungus isolated from sphagnum moss.[61] It has been observed growing free on grains, and Benham and Kesten[12] demonstrated its ability to grow on barberry and carnations; in the latter a bud rot was produced, an interesting example of infection of hosts as diverse as plants and man. It seems highly probable, therefore, that the fungus lives a saprophytic existence in nature, occasionally setting up an infection in man when mechanically introduced into the tissues.

Diagnosis. The diagnosis of sporotrichosis is established by demonstration of the fungus. The cigar-shaped parasitic cell is gram-positive, but may be found only rarely in gram-stained pus smears. These forms are relatively infrequent in human material, and sporotrichosis is not excluded by failure to find them.[49] The fungus is readily cultivated on Sabouraud's medium from pus aspirated from unopened lesions. Rats are highly

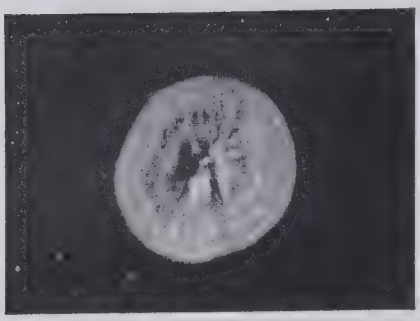


Figure 197. Giant colony of *Sporotrichum schenckii* on Sabouraud's agar, 3 weeks old. × 2.

susceptible to infection, and rat inoculation is of considerable diagnostic value. Male white rats are inoculated intraperitoneally; a pronounced orchitis occurs, and there is generalized peritonitis with minute nodules on all the peritoneal surfaces. The cigar-shaped cells are present in abundance in the rat lesions and may be found readily in gram-stained smears.

Immunity. There is an immune response to infection manifested by the appearance of agglutinins (for spores) and complement-fixing antibodies. These are of some diagnostic value though somewhat nonspecific in that serums from persons with thrush or actinomycosis will give positive reactions. A cutaneous test with *sporotrichin,* a preparation analogous to tuberculin, is of similar specificity.

THE DERMATOPHYTES[38, 69, 183]

By far the most common type of fungous disease of man is dermatophytosis (dermatomycosis), a superficial infection of the keratinized epidermis and keratinized epidermal appendages, the hair, hair-sheaths, and nails, the severity of which is dependent for the most part upon the location of the lesion and the species of fungus involved. Though certain other fungi, notably Candida, produce clinically similar disease, a more or less homogeneous group of fungi, the dermatophytes, is responsible for the great majority of cases. The ability of these microorganisms to invade and parasitize the cornified tissues is closely associated with, and dependent upon, their common physiological characteristic, the utilization of the highly insoluble scleroprotein keratin. The utilization of keratin is biologically rare and is shared by the dermatophytes only with a few species of saprophytic fungi and certain insects including the clothes moth (Tinea), the carpet beetles (Dermestes), and the biting lice (Mallophaga).

Though the various species of dermatophytes produce infections that are clinically characteristic, on the one hand single species may produce different types of disease, and on the other, infections that are very similar or essentially identical may be produced by different species. Furthermore, other conditions such as chemical dermatitis, neurodermatitis, and certain types of allergy may closely simulate dermatophytosis, but do not, of course, respond to treatment with fungicides. Demonstration of the causative fungus by direct microscopic examination of pathological material or by isolation and culture is, therefore, highly desirable, especially in all cases presented for treatment.

As noted earlier, the antibiotic griseofulvin has proven to be a highly effective chemotherapeutic agent for the treatment of the dermatomycoses,[136, 181, 193] but cure commonly requires prolonged (3–10 weeks) administration by mouth. The antifungal effect of the antibiotic is reflected in morphological changes in the infecting fungus, both *in vivo*[132] and *in vitro*.[152]

The dermatophytes are mold-like fungi grouped with the Fungi Imperfecti;[186] ascospore formation has been reported by some workers but has not been unequivocally established except in the case of *Microsporum gypseum* which has been shown to be a perfect fungus for which the name *Nannizzia incurvata* has been proposed.[171] These organisms differ from most other pathogenic fungi in that the cells are multinucleate, usually containing four to six nuclei, and division is amitotic. Arthrospores, chlamydospores, and the individual cells of

The Common Dermatophytes

			SPECIES	DISEASE IN MAN	GEOGRAPHICAL DISTRIBUTION
Invading the hair and hair follicles	Small spore varieties		*Microsporum audouini*	Prepuberal ringworm of the scalp; suppuration rare	Commonest in Europe, producing about 90% of infections; about half infections in U. S.
			Microsporum canis	Prepuberal ringworm of scalp and glabrous skin; suppuration not infrequent; kerion occasional	Uncommon in Europe; responsible for about half the infections in U. S.
			Microsporum gypseum	Prepuberal ringworm of the scalp and glabrous skin; suppuration and kerion common	Relatively rare in U. S.; common in South America
	Large spore varieties	Endothrix type	*Trichophyton tonsurans*	Ringworm of the scalp and smooth skin; sycosis; onychomycosis; suppuration common, the hair follicles are atrophied	Common in Europe, Russia, Poland, Italy, Near East, but uncommon in U. S.
			Trichophyton schoenleinii	Favus, in both scalp and smooth skin; onychomycosis; suppuration is the rule and kerion frequent	Common in Europe and the Far East; rare in U. S.
		Ectothrix type	*Trichophyton mentagrophytes*	Commonest cause of intertriginous dermatophytosis of the foot ("athlete's foot"); ringworm of the smooth skin, suppurative folliculitis in scalp and beard	Ubiquitous
			Trichophyton verrucosum	Ringworm of the scalp and smooth skin, suppurative folliculitis in scalp and beard	Ubiquitous
			Trichophyton megnini	Sycosis is the most common lesion; infection of smooth skin and nails	Sporadic distribution
Not invading the hair and hair follicles			*Epidermophyton floccosum*	Cause of classic eczema marginatum of crural region; causes minority of cases of intertriginous dermatophytosis of foot; not known to infect hair and hair follicles	Ubiquitous, but more common in tropics
			Trichophyton rubrum	Psoriasis-like lesions of smooth skin; onychomycosis, mild suppurative folliculitis in beard	Common in Far East, tropics and southern U. S.
			Trichophyton concentricum	Commonest cause of tinea imbricata; infection of hair and nails uncertain	Common in South Pacific islands, Far East, India, Ceylon; reported in South America; does not occur in U. S.

fuseaux are also multinucleate, but aleuriospores contain but a single nucleus. The group is a homogeneous one, immunologically as well as morphologically and physiologically, and the fungi comprising it are more closely related to one another than to other fungi. The affinities of the group to the other fungi are not clear, however, and the dermatophytes have never been satisfactorily oriented in a general system of fungus classification; they constitute a group somewhat apart and are usually neglected by botanists.

Differentiation of Genera and Species. The differentiation of species within the group is somewhat uncertain in a general sense. The classification of Sabouraud, presented in 1910 and somewhat modified by him later, is the most generally used. It is based on both the clinical character of the infection and on colonial morphology, including pigmentation, the morphology of the reproductive and vegetative structures being a secondary matter. The species so differentiated are sound natural groups in spite of objections raised by some systematists, and the classification and differentiation are practical and workable.

It is to be noted, however, that colonial morphology is readily altered by continued culture on artificial mediums. In consequence many stock cultures of dermatophytes are atypical to a greater or lesser extent. Such variation is especially prone to occur on maltose "proof" agar, and Sabouraud recommends a

milieu de conservation, the same medium but lacking sugar, which does not bring out the differential characters of all species; growth on it is much slower, but it has the virtue of indefinitely postponing pleomorphic changes. Differential characteristics reappear, of course, on transfer to the maltose *milieu d'épreuve*.

Differential characteristics. Very many different kinds of dermatophytes have been described, some estimate as many as 200 or more, but many "new" species have been inadequately studied and often represent minor variants of established species. Primary differentiation into the three genera, Trichophyton, Epidermophyton, and Microsporum, is morphological and based on colonial morphology and the character of the macroconidium. A number of physiological studies have been carried out,[170] and it has been found that certain species or strains are distinguished by requirements for vitamins or amino acids. It has been suggested[62] that these might provide a basis for identification, but so far at least physiological characteristics have not been as useful for the differentiation of these forms as they often are for the characterization of bacteria.

These fungi may also be separated into two groups on the basis of the character of growth in the host tissue, *i.e.*, those which invade the hair and hair follicle and those which do not.

While parasitic on the skin and its appendages, the thallus is differentiated only into hyphae and arthrospores; the latter, arising by fragmentation of the mycelium are to be regarded as oidia, as indicated earlier, though usually termed "spores" by dermatologists. The variety of differentiated structures, including aleuriospores, spindle-spores or fuseaux, and vegetative structures such as spirals, pectinate bodies, nodular organs, and racquet mycelium, appear on cultivation on artificial mediums. The differences are no doubt a consequence of the parasitic vs. saprophytic state.[7]

In the first group there are, for present purposes, two genera which differ with respect to size and arrangement of the spores formed in the hairs. The genus Microsporum includes the small spore type (3 to 4 μ in diameter) and the genus Trichophyton, the large spore type (7 to 8 μ in diameter). This distinction is not absolute for the very common species, *Trichophyton mentagrophytes*, forms small spores. Furthermore, the spores differ in arrangement. While the mycelium of Microsporum grows within the hairs, spores are formed only outside of the hair and occur in irregular clusters in a kind of mosaic arrangement. The spores of Trichophyton, on the other hand, occur in chains inside or outside the hair.

Further differentiation of Trichophyton is made on the basis of location of the growth with respect to the hair.[92] The

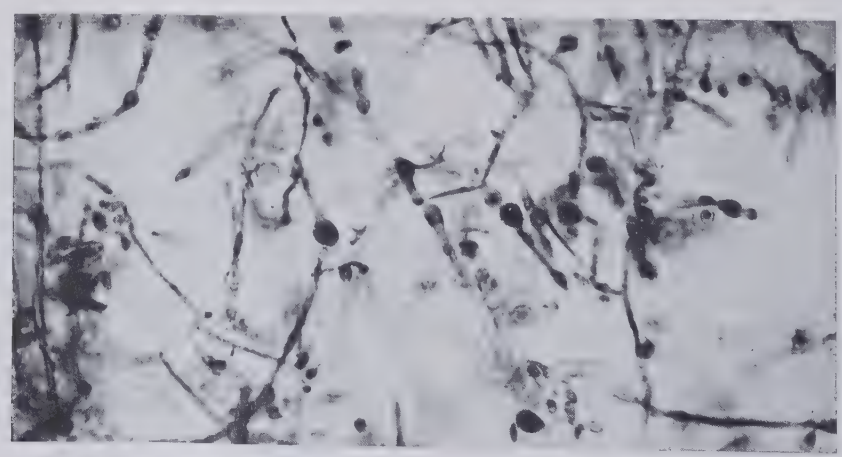


Figure 198. Chlamydospores, *Microsporum audouini*. Mounted in Amann's lactophenol cotton blue solution; × 420.

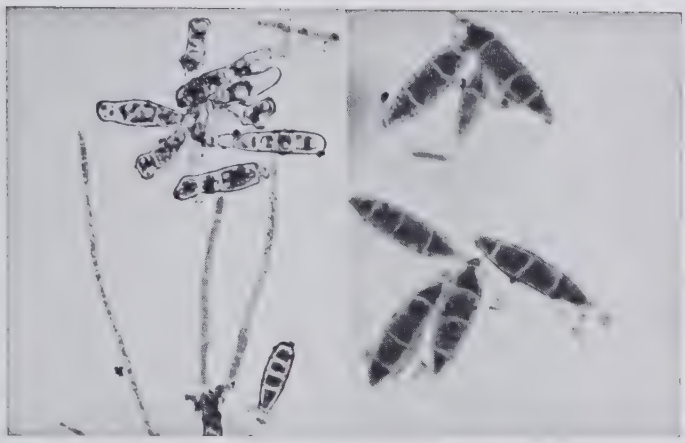


Figure 199. *Left,* blunt end fuseaux, *Epidermophyton floccosum.* Note the attachment to the mycelium. Mounted in Amann's lactophenol cotton blue solution; × 420. *Right,* pointed fuseaux, *Microsporum gypseum.* Mounted in Amann's lactophenol cotton blue solution; × 420.

endothrix type (sometimes called Endotrichophyton) grows within the hair, and mycelium and chains of spores are found there. The *neo-endothrix type* grows for the most part within the hair, but in some hairs growth occurs on the surface. Finally, the *ectothrix type* (sometimes called Ectotrichophyton) forms spores only on the surface of the hair though the mycelium grows inside.

It will be clear that differentiation of these genera may be approximated by, first, the clinical character of the disease and, second, by direct microscopic examination of epilated infected hairs, but identification is possible only by culture. In general Trichophyton infections show a characteristic tendency to produce an inflammatory reaction with deep infiltration of the skin that is rarely produced by Microsporum infections; this difference is of some value in distinguishing between Microsporum infections and infections of the scalp with small-spore ectothrix Trichophyton. The animal strains of Microsporum such as *Microsporum canis* and *M. gypseum,* however, also elicit inflammatory reactions.

Species of Epidermophyton invade the superficial layers of the skin, and the fungi are found in scales of the epidermis taken from the periphery of the lesion as articulated filaments of mycelium breaking up into chains of round to oval arthrospores. Endotrichophyton characteristically grows within the epithelium between the superficial and deeper layers but does not invade the corium; these forms are very difficult to cultivate.

Pathogenicity. At present the dermatophytes are known as parasites and are usually assumed to be obligate parasites either of man alone or of man and animals. The majority grow readily on laboratory mediums and also grow on such substrates as cereal grains, shed hair, horn debris, and sterilized fragments of straw in moist tubes and will remain viable in litter containing such materials for 2 to 3 years. If protected from dryness they may live on the wooden floors of shower rooms, dressing cabins, mats, etc., for a considerable time. Further, various dermatophytes have been isolated from soil and air,[51, 54, 56, 134] and it is possible that this and other dermatophytes may live a saprophytic existence, unrecognized because of a variety of differentiated structures, and it seems probable that at least a saprophytic stage may occur in the transmission from one animal to another.

The dermatophytoses show, in many instances, a pronounced age and sex distribution, and there is some difference in the geographical distribution of the various species. Common ringworm or "gray patch" of the scalp is confined to the young, occurring more often in boys than in girls, and is rare after puberty. Others, such as Epidermophyton species, occur for the most part in adult males. The distribution of intertriginous dermatophytosis of the feet, commonly known as "athlete's foot," in the young adult male is probably in large part an expression of risk. *M. canis* is more common in this country than in Europe, and

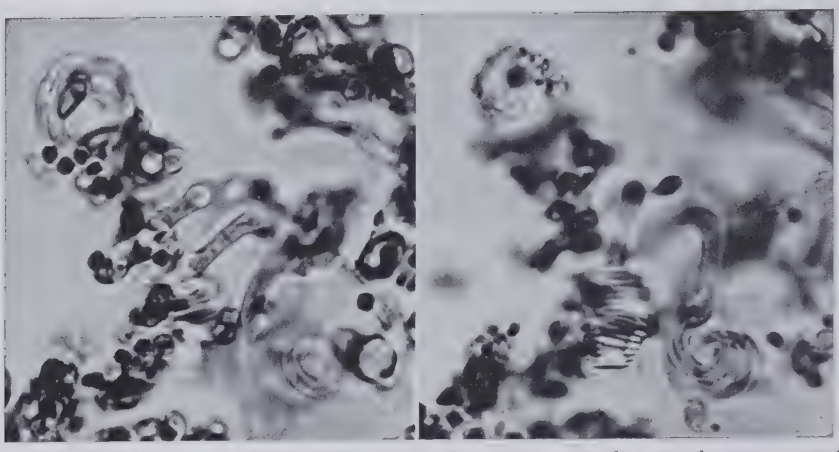


Figure 200. Spiral hyphae, *Trichophyton mentagrophytes* var. *granulosum*. The same spiral appears in different planes of focus. The small dark bodies are microconidia. Mounted in Amann's lactophenol cotton blue solution; × 950.

the reverse is true of *T. schoenleinii,* while *T. concentricum* is well known in the tropics and certain parts of the Far East but is rare in temperate climates.

Some dermatophyte species appear to be so closely adapted to man that they are unable to infect lower animals and human infection is transmitted by contact.[43, 104] Others not only produce infections in experimental animals, but animals such as the cat and dog are natural hosts, and human infection may be acquired from them. The reservoir of animal-type dermatophytes is of considerable epidemiological importance[97, 113, 116, 135]; one survey showed for example, 37 per cent of cats and 22 per cent of dogs examined at random in this country were infected. There is also a high degree of specificity as to the tissues attacked. While, as indicated earlier, these fungi are well adapted to parasitize the horny layer of the epidermis, they appear to be unable to invade and infect other organs of the body. The intravenous injection of Microsporum spores or emulsions of virulent *T. mentagrophytes* does not produce an infection of the internal organs of susceptible animals; rather, the microorganisms introduced tend to become localized in the skin and to develop where it is damaged as by scarification.

Growth of the fungus in the skin and hair is more or less equal in all directions, and the lesions produced tend to have a circular form. For this reason the Greeks named the disease herpes, a term which still persists though modified as *herpes tonsurans, herpes circinatus,* or *herpes desquamans* to distinguish the dermatophytoses from herpetic infection of virus etiology. The Romans associated the lesions with lice and named the condition *tinea,* meaning any small insect larva. This name is likewise in common use. The English *ringworm* is, of course a combination of the Greek and Roman terms.

With any species of dermatophyte, infection begins in the horny layer of the epidermis. Those which infect the hair follicles, hair, and nails soon invade these structures, often producing little more than a scaling of the epidermis. Those which remain in the epidermis affect the drier parts of the skin, including the palmar and plantar surfaces, or the moister regions in the inguinocrural fold and the interdigital spaces. Thus a wide variety of clinical types may be observed, but the differences are more apparent than real for the pathological changes are fundamentally the same in all types.

Infection of the dry glabrous skin. The formation of characteristic concentric rings of inflammatory reaction is more evident in infections of the smooth skin than in hirsute areas. Two types

Figure 201. *Hyphae sporiferae* (*thyrsi sporiferae, thyrsi*) in culture of *Trichophyton purpureum*. The attachment of aleuriospores, directly or by very short stalks, to the hyphae is evident. Unstained preparation; × 450. (Rothman.)

of lesion are produced, the one dry and scaly and the other vesicular. The first begins as a small elevated area of inflammation which spreads, the margin remaining red and sometimes slightly swollen while the central area becomes covered with small scales; spontaneous healing occurs in the center as the circinate margin advances.

In the second type vesicles appear irregularly or immediately back of the advancing hyperemic and elevated margin; a crust is formed, followed by spontaneous healing in the center to leave a more or less pigmented area. The lesion may become pustular with invasion of the hair follicles. In either case the fungi are most numerous at the margin of the advancing lesion, and scales or vesicles from this region are best suited for study and isolation of the microorganism.

Infection of the palmar and plantar surfaces (the latter is the more common) is ordinarily secondary to infection of the interdigital spaces. Vesicles are formed with the thick horny layer of the sole separated from the epidermis beneath; the vesicle may be filled with fluid but soon dry. When the top peels off it leaves a pink area surrounded by a border of loosened and peeling skin. The formation of secondary vesicles and continuous repetition of the process produces widening areas of pink or reddish skin, the dyshidrotic form of the disease. The formation of fissures, common in the interdigital areas, may also occur on the sole of the foot. A dry, hyperkeratotic type of lesion without vesiculation is produced in the chronic infections.

Infection of the epidermis of the principal folds. In these regions there are also two main types of dermatophytosis, the one, *eczema marginatum*, chiefly confined to the crural fold, and the dyshidrosiform intertriginous mycoses of the hands and feet. In the former a small round swollen area of inflammation appears which spreads, producing at first a circinate lesion which later becomes irregular owing to differences in rate of spread. The actively developing margin is an inflamed area in which there are numerous small vesicles filled with a serous exudate. In most instances the infection begins on the thigh where it is in contact with the scrotum, and spreads rapidly, usually involving the inner thigh and sometimes the gluteal and pubic regions. Pruritus is a common symptom.

In the interdigital areas, usually of the toes (moniliasis is the more common in the drier areas between the fingers), the epidermis is macerated or shows circinate scaling. Small eroded patches are common. Vesicles are rarely seen because they rupture early, leaving circular or polycyclic epithelial fringes. Deep within the fold the epidermis is membranous and swollen, fissures form, and the macerated epithelium is shed as moist, white scales.

Infection of the hair and hair follicles. Invasion of the hair follicles and hair produces two general types of lesions. The most distinct and first to be differentiated clinically is that of *favus*, produced by *T. schoenleinii* and distinguished by the formation of the *favic scutulum*. Beginning with a small spot or scaly inflammation, crusts appear as yellow points about the hair follicles. The hyphae and spores form a ring about the follicular orifice, and a leucocytic infiltration produces an intraepidermic pustule. A yellow disk appears at the base of the pustule which enlarges with the lesion to produce the cup-shaped yellow crust known as the scutulum, which in time often becomes as large or larger than a pea. On the upper or concave surface the epidermis is intact and the hair is brittle, lusterless, and gray, but not necessarily broken, while the epidermis below is infiltrated and sclerosed. Exfoliation of the horny layer occurs, some of the upper layer of the scutulum being removed. The scutulum itself consists of a mass of fungous mycelium in an enlarged hair follicle; the center surrounding the hair is a tangle of broken hyphae and spores inside an area of regular hyphae in the epidermis, and at the periphery the hyphae are perpendicular to the

surface. Infection of the hair begins at the root and some time, usually 3 to 4 months, is required for the fungus to grow up through the hair to produce the dull, powdery gray, dry appearance characteristic of favus. The hair remains, though seldom more than a few centimeters in length, for several years before being shed through scar formation in the follicle, but in time it loosens, falls out, and may not return, resulting in patches of permanent alopecia. Section of infected hairs shows air spaces, presumably left by dead hyphae; relatively few hyphae remain, and the hair structure is clearly visible.

The second type of infection includes ringworm of the scalp or *tinea tonsurans,* which is much the more common in this country, and infection of the beard. It may be subdivided into Microsporum infection, endothrix Trichophyton infection, and suppurative infections usually with ectothrix Trichophyton.

In Microsporum ringworm the fungus invades the hair follicle to form a mass of mycelium between the hair and the epithelium. Some hyphae penetrate the hair and grow downward, while others grow on its surface to form the characteristic layer of spores. The lesion begins as a small erythematous area. Within a few days it pales and becomes scaly, and the hair assumes the characteristic grayish, discolored appearance, becomes weakened, and breaks off a few millimeters above the scalp. The lesion spreads in the characteristic ring form and may coalesce with other infected areas produced by autoinoculation to give an irregular area of infection in which all the hairs are affected. There is little inflammatory reaction.

Trichophyton ringworm differs from the Microsporum infection in a number of respects. While infection of the hair follicle and hair follows essentially the same course, the lesions are small with often only two or three affected hairs and all the hairs within a given lesion are not affected, and the lesions are multiple, numerous, and scattered over the scalp. The infected hairs break off sharply at the follicular orifice in *T. acuminatum* infections, and somewhat above to leave stumps in *T. crateriforme* infections. There is somewhat more inflammatory reaction than in microsporiasis and often spread of the infection to the glabrous skin.

Infections with ectothrix Trichophyton are distinguished clinically by a more marked inflammatory reaction and often suppurative folliculitis. There is an infiltration of serum and leucocytes into the skin of the affected area and an infiltration of the deeper tissues. In areas of contiguous suppurative folliculitis an indolent cutaneous-subcutaneous infiltration called *kerion* is formed which is boggy on palpation, pus oozing from the follicles on pressure. Suppurative lesions of the beard are termed *sycosis.* Suppurative infection and kerion may, of course, occur with other species, including endothrix Trichophyton and Microsporum of animal origin.

It was observed by Margarot and Devéze in 1925 that infected hairs and fungus cultures show fluorescence in ultraviolet light.* This empirical observation has proved to be of very considerable practical value, especially in Microsporum ringworm of the scalp, although hairs infected with *M. gypseum* often do not have this property. It is generally agreed that all hairs infected with Microsporum show a brilliant greenish fluorescence and those infected with *T. schoenleinii* a greenish but less brilliant fluorescence, both distinct from the bluish tint of normal skin. There appears to be some disagreement with respect to Trichophyton infection; it is reported by Davidson and Gregory[31] that in infection with some species the hairs fluoresce but in others they do not, while Lewis

* "Black light," commonly known as Wood's light because the radiation is filtered through Wood's nickel oxide glass which holds back almost all the visible rays but passes the longer ultraviolet rays.

Figure 202. Spiral hyphae in culture of *Trichophyton mentagrophytes* var. *gypseum.* Unstained preparation; × 450. (Rothman.)

Figure 203. Chlamydospore (*upper left*) and racquet mycelium (*lower right*) in culture of *Microsporum audouini*. Unstained preparation, oblique lighting; × 950. (Rothman.)

and Hopper state that all endothrix Trichophyton species show a dull bluish fluorescence of the infected hair though hair infected with ectothrix Trichophyton does not fluoresce. It has been found that the fluorescence is due to the presence of a substance in the hair which can be extracted either with warm water after preliminary ether extraction or with dilute alkali.[31, 137]

Infection of the nails. Infection of the nails, *tinea unguium* or *onychomycosis,* may be either primary or secondary to an epidermal lesion. The infected nails are usually opaque, lusterless, yellowish and friable, and varying degrees of dystrophy are found. The infection ordinarily begins under the free border or along the lateral margin of the nail, producing an opaque, yellowish white irregular spot beneath the central area. This may develop into a thick spongy friable layer, readily detachable. As the nail plate separates from the bed, yellowish or white longitudinal streaks usually appear, it becomes thin and brittle and breaks off; sometimes the entire nail plate is lost. When the surface of the nail is attacked early, it becomes fissured and soft and wears away. In general, there is no marked inflammatory reaction. The disease is of long duration, and may last 10 years or more, and there is little if any tendency to spontaneous healing. The disease is usually due to Trichophyton, but Microsporum and Epidermophyton infections have been observed.

Immunity. Though an acquired immunity to infection has been demonstrated in experimental animals by a number of workers, the status of an effective immunity in man is uncertain. There have been some enthusiastic reports of the efficacy of vaccine therapy which are, perhaps, open to question. Much of the work that centers about local immunity appears to be uncritical, *viz.,* in many cases remarkable results have been reported but have not been regarded by immunologists generally as contributing materially to a solution of the general problem of local immunity. Hypersensitivity, however, is a common, though not invariable, manifestation of the immune response to dermatophyte infection, and desensitization procedures appear to have definite therapeutic value in certain cases.

Hypersensitivity is manifested in two ways. One of these is the appearance of secondary, nonparasitic lesions on parts of the body remote from the infection. These are termed "ids," in a general sense *mycid* or *dermatophytid,* and more specifically *microsporid, trichophytid,* and *epidermophytid.* The mycid takes the form of a symmetrical eruption over relatively large areas, usually of the trunk, as a rash. The eruption may be vesicular with sterile content, papular, or lichenoid and is sometimes localized at the follicular pores. A rather frequent occurrence is a sterile vesicular eruption on the hands secondary to infection of the feet. It is generally believed that spores or bits of mycelium from the infection lesion enter the blood stream and are eventually deposited in the skin where they induce the local allergic response with destruction of the fungous elements. This view is based in part on a number of successful isolations of

these fungi from the blood stream during the development of mycids. It is not known what part, if any, soluble substances liberated by the dissolution of the fungi play in the phenomenon.

Hypersensitivity may also be demonstrated by the injection or application of preparations of dermatophyte cultures analogous to tuberculin and called *trichophytin.*[29] The local and constitutional reactions that may be produced with trichophytin are much the same as those to tuberculin. The test does not differentiate between Microsporum and Trichophyton or their species, for all appear to contain a common or very closely related antigen. The utility and reliability of the trichophytin test in diagnosis form a subject of conflicting opinion. It is maintained by some that the test is a valuable adjunct to other methods of diagnosis when used with the proper precautions; others, however, regard the test as of questionable value because, on the one hand, infection does not always result in sensitization, and, on the other, a positive reaction may only indicate a past infection unrelated to the condition in question.

Characteristics of Dermatophyte Species. The characteristics of the more important species of dermatophytes may be considered briefly:

Microsporum audouini (*Sabouraudites audouini, Closteroaleuriosporia audouini*). This fungus is the classic etiological agent of common prepuberal ringworm of the scalp; it is relatively more common in Europe, where it causes perhaps 90 per cent of the infections, than in this country where it is found in about half the cases. An inflammatory reaction is produced only occasionally, suppuration is uncommon, and kerion very rare. The trichophytin reaction is usually slight or absent.

Microscopic examination of the epilated hair stub shows hyphae within the hair and external irregular clusters of spores. The colony on maltose agar begins as a white fluffy mass which develops a central elevation and radial furrows and becomes grayish to buff in color as it matures. Aerial growth is scanty. About 2 weeks' incubation suffices for full development of colonial character. In slide culture fuseaux are rare and conidia few, but pectinate bodies and racquet mycelium may be formed. Experimental animals are highly resistant to infection.

Microsporum canis (*Microsporum lanosum, Microsporum caninum, Sabouraudites lanosus, Closterosporia lanosa*). *M. felineum* is regarded by some as a separate species and by others as only a variety of *M. canis*. The fungus is the cause of half or more of the cases of ringworm in children in this country but is found in only a small minority of cases in Europe. It differs from *M. audouini* in that it is a natural parasite of lower animals—it was originally found in the dog—and children may contract the disease from infected kittens and puppies as well as from one another. Infections of the smooth skin are common and produce a circinate lesion with a vesicular border and mild erythema. In the scalp there is a mild inflammatory reaction, and the infection is not infrequently suppurative; kerion occurs in 2 to 3 per cent of the cases. The trichophytin reaction is usually strongly positive.

In the epilated hair the fungus is indistinguishable from *M. audouini*. Colonial growth is somewhat more rapid, and a yellowish pigment is produced, the mature colony being tan and the medium a yellowish color. Both radial and concentric striations or furrows are often present, and there is an abundant and wooly aerial growth. Pleomorphism after 4 to 5 weeks' incubation is the rule. In slide culture fuseaux are

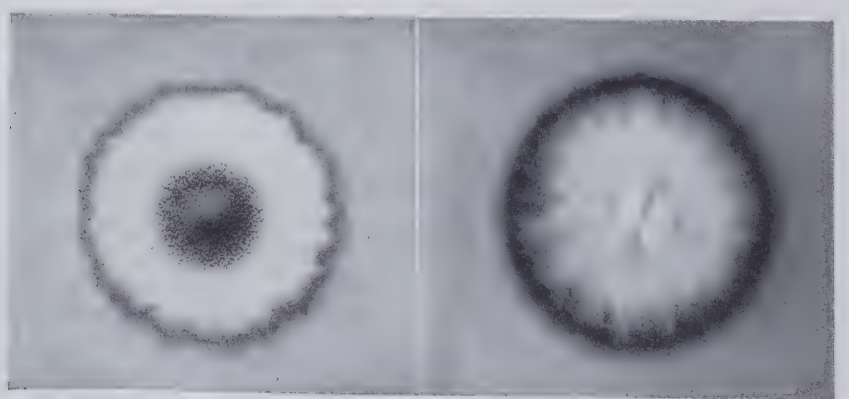


Figure 204. Dermatophyte colonies on Sabouraud's agar. *Left, Microsporum audouini.* The colony is grayish with a buff center. *Right, Microsporum canis.* The colony is a light pinkish tan with a yellow border, natural size.

abundant in the center of the growth; other structures such as conidia, racquet mycelium, and pectinate bodies are not striking. Kittens, puppies, rabbits, and guinea pigs are readily infected experimentally.

Microsporum gypseum (*Microsporum fulvum, Closterosporia fulva, Achorion gypseum*). Like *M. canis*, this is an animal parasite and is one cause of dog favus. The disease in the scalp is similar to that produced by *M. canis* but with more marked inflammatory reactions as a rule, and suppuration and kerion are common. The smooth skin may be infected. The trichophytin reaction is strongly positive.

In infected hairs the spores tend to a linear arrangement in the early stages of the disease, and later the picture is indistinguishable from that produced by *M. audouini* and *M. canis*. Colonial morphology is highly characteristic; a central umbo, which may be white, appears early and the colony is a cinnamon to pinkish brown. Concentric furrows may be present. In slide culture fuseaux are numerous, and racquet mycelium and nodular organs may be found together with moderate numbers of conidia. The rabbit, cat, and guinea pig may be infected.

Trichophyton tonsurans (*Trichophyton acuminatum, Trichophyton crateriforme, Trichophyton cerebriforme, Chlamydoaleuriospora crateriformis, Trichophyton flavum, Neotrichophytum flavum, Trichophyton sabouraudi, Aleuriospora acuminata*). This fungus is one of the common causes of trichophyton ringworm of the scalp in Europe. Like other trichophytoses, *T. acuminatum* infections, though more common in children, persist into adult life. Infection of the smooth skin occurs with the formation of a circinate lesion with vesicular border. Infection of the nail occurs. The trichophytin reaction is positive. The fungus is of the endothrix type and chains of spores are found within infected hairs. Three colonial types occur as variants which have been distinguished in the past as separate species. One, designated *T. acuminatum*, shows a flattened conical peak in the center of the colony with radiating furrows and peripheral folds. A second type, *T. crateriforme*, has a colony which is sunken in the center with a raised ring at the periphery. The colony of the third type, *T. cerebriforme*, is crateriform at first, but the depression tends to fill in, and the folds become prominent. All are creamy white in color but some strains may be yellowish or violet, and the surface is fine and powdery.

In slide culture there is an abundance of round to oval microconidia, borne laterally or on the tips of hyphae, as thyrsi and *en grappe*. Chlamydospores are present but there is no characteristic predominance of vegetative structures. A mild localized infection may be produced in the guinea pig.

Trichophyton schoenleinii (*Achorion schoenleinii, Arthrosporia schoenleinii, Grubyella schoenleinii*). Favus is prevalent in Eastern Europe and the Far East but relatively uncommon in this country. It is characterized by the formation of the favic scutula described earlier. The formation of scutula is the rule on the scalp, and they may be found in infection of the skin, or the skin lesions may be vesicular or scaly. Infection of the nails with *T. schoenleinii* produces severe dystrophy of the nail plates. Most human cases are infections with this species, but related forms produce disease in animals, such as *T. quinckeanum* of mouse favus, and are occasionally transmitted to man.

Infected hairs show large spores in chains and coarse filaments within the shaft and, characteristic of this infection, air bubbles left by degeneration of the fungous elements within the hair. The distribution of the fungus in the scutulum has been described earlier. Growth in culture is slow, and usually 3 weeks are required for development of the colonies. They are yellowish and waxy in appearance at first, and later usually become wrinkled and whitish with aerial mycelium. The fungus is highly pleomorphic in slide culture, showing various irregular bodies, swollen and distorted hyphae, etc., but the branched structure known as the favic chandelier is readily found and diagnostic, for it is not formed by other species of dermatophytes. The fungus is pathogenic for mice, and nodular lesions of the lungs or peritoneum may be produced in the rabbit by intravenous or intraperitoneal inoculation respectively.

Trichophyton mentagrophytes (*Trichophyton gypseum, Trichophyton asteroides, Trichophyton pedis, Trichophyton interdigitale, Trichophyton niveum, Ectotrichophyton mentagrophytes, Sabouraudites asteroides, Aleuriospora rosacea, Ctenomyces mentagrophytes, Spiralia mentagrophytes*). This fungus is by far the commonest cause of dermatophytosis of the foot (athlete's foot) and also produces tinea circinata and follicular infection of the scalp with kerion as well as onychomycosis. The number of synonyms is due in part to the pleomorphism of the fungus; the first five given above have been commonly used in the past to denote different but closely related species. It is becoming increasingly apparent, however, that these forms lie in continuous series between the granular *T. gypseum* varieties and the floccose type of growth characteristic of *T. interdigitale*. Here we shall follow the suggestion of Emmons and include them all in the single species *T. mentagrophytes*. It may be noted also that *T. granulosum* and *T. felineum* are so closely related to *T. mentagrophytes* that they can hardly be regarded as more than varieties of that species. Trichophytids associated with intertriginous infection are usually due to this fungus. The trichophytin reaction is positive.

Infection of the hair is of the ectothrix type and, as noted earlier, the fungus is a small-spore type. The hair is usually not broken, and the external spores are relatively few in number and in linear arrangement in contrast to Microsporum. In scales and macerated tissue hyphae and spores are found but are not characteristic. There are

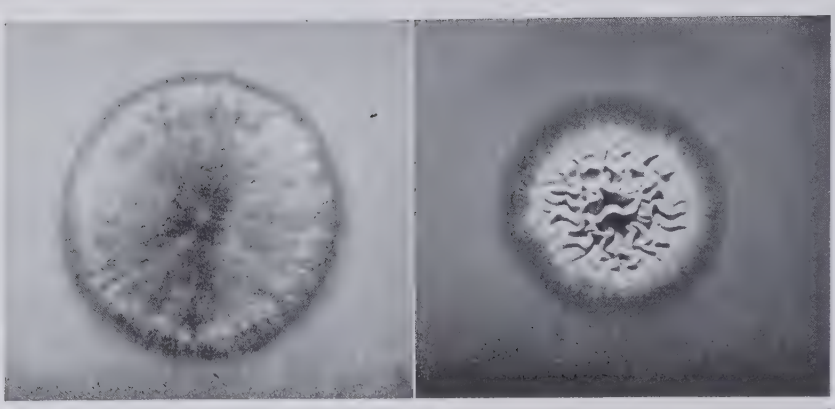


Figure 205. Dermatophyte colonies on Sabouraud's agar. *Left, Microsporum gypseum.* The colony is a uniform pinkish tan color. *Right, Trichophyton schoenleinii.* The colony is a very light pinkish yellow and characteristically wrinkled.

four general types of growth with respect to colonial morphology. (1) The type most often found produces a fluffy colony that becomes flat, velvety, and yellowish buff in color as it matures. Slide cultures are characterized by the presence of spirals. Nodular organs, racquet mycelium, pectinate bodies, chlamydospores, and fuseaux may be found together with conidia in thyrsi and *en grappe*. (2) The white fluffy type with aerial mycelium is often called *T. interdigitale*. Fuseaux and spirals are usually absent in slide cultures; the aerial mycelium is largely vegetative with conidia in small clusters, and nodular organs and racquet mycelium may be found. (3) The granular, powdery, light tan to yellow colony is the *T. gypseum* type of growth. The surface is velvety and tends to become fluffy with age. Slide culture shows few spirals but numerous fuseaux and conidia *en grappe* and as thyrsi. (4) The white colony of the *T. niveum* type begins as fluffy but becomes compact and shows surface irregularities with maturity. The morphology of the fungus in slide culture closely resembles that of the *T. interdigitale* type. Dogs, cats, rabbits, and guinea pigs may be infected; the granular types of the fungus are the more virulent.

Trichophyton megnini (*Trichophyton roseum, Ectotrichophyton megnini, Megatrichophyton megnini, Megatrichophyton roseum, Aleuriospora rosacea*). This fungus infects the epidermis, nails and hair follicles, usually without marked inflammation, and lesions are most common in the beard. It is common in the north of England and endemic in most of Europe. The infection is spread through barber shops.

Infection of the hair is of the ectothrix type, the spores are arranged in chains and, in contrast to *T. mentagrophytes*, are large. The lesion on the skin is usually dry and scaly, and the fungus elements may be found in scrapings. The colony is velvety, pure white when young and gradually turning a pale rose with maturity while the under side becomes violet. Chlamydospores and conidia, *en grappe* and as thyrsi, are found in slide culture, but the morphology is not characteristic. Infection may be produced in the guinea pig.

Epidermophyton floccosum (*Epidermophyton inguinale, Epidermophyton cruris, Trichophyton inguinale, Trichophyton cruris, Closterosporia inguinalis, Fusoma cruris*). The fungus is the cause of classic eczema marginatum (dhobie itch) and is ubiquitous in distribution though generally regarded as considerably more prevalent in the tropics. It may be disseminated in semi-epidemic form. It is also the cause of a minority of the intertriginous dermatophytoses of the feet. This fungus has never been observed to infect the hair follicles. The trichophytin reaction is practically always negative.

In scales taken from the advancing margin of the lesion, hyphae and chains of spores may be found, in large amount when the scaling is profuse. Growth is relatively slow. The colony is characteristically an olive drab color, aerial mycelium is usually scant, and the surface is velvety and irregularly folded with radial furrows. Pleomorphic growth, manifested as the appearance of white tufts, appears relatively early. Blunt end fuseaux are present in groups in slide cultures of the fluffier strains, and there are many chlamydospores, but structures other than racquet mycelium are seldom found. The fungus is only slightly pathogenic for the guinea pig.

Trichophyton rubrum (*Epidermophyton purpureum, Trichophyton purpureum*). This fungus is one of the common causes of intertriginous infection of the foot. It invades the skin to produce a typical psoriasis-like lesion, mildly inflammatory with the affected area dull red and scaly and the skin thickened. It is not infrequently found in onychomycosis, and a mild suppurative folliculitis may be produced in the beard. The fungus is found in the Far East and is common in the tropics and the southern part of this country. The trichophytin reaction is negative, or faintly positive.

The colony is at first raised, fluffy, and white. Radial grooves are formed, and the typical rose-purple color at the back of the colony develops

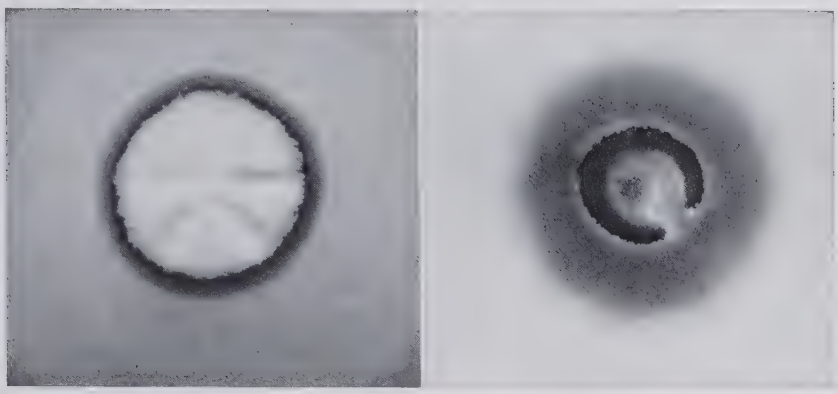


Figure 206. Dermatophyte colonies on Sabouraud's agar. *Left, Trichophyton rubrum.* The colony itself is white with sometimes a very pinkish tinge, and the fungus derives its name from the deep rose-purple pigment on the bottom of the colony; here the pigment has diffused slightly to give a rim of color about the colony. *Right, Trichophyton tonsurans.* In this case the deep violet pigment is in a ring, and the remainder of the colony is rose and tan.

early and gradually diffuses into the colony to give a white center on a tinted background. Strain variation in the formation of aerial mycelium results in considerable variation in color as viewed from above. The morphology in slide culture is not characteristic, consisting largely of vegetative hyphae with occasional racquet mycelium, conidia, fuseaux and chlamydospores.

Trichophyton concentricum (*Endodermophyton concentricum*). This fungus is the commonest cause of *tinea imbricata* (tokelau, Malabar itch), a ringworm of the glabrous skin found in the South Pacific Islands, the Far East, and in India and Ceylon. Recently it has been reported from South America but apparently does not occur in this country. The closely related species, *Endodermophyton indicum* and *E. tropicale,* are frequently found in Malaysia and Ceylon respectively. The name *E. tropicale* is used by many as a synonym of *T. concentricum.* The fungus grows between the superficial and deeper layers of the skin with little or no inflammation to produce a dry scaling lesion. The picture of the disease is highly characteristic; a circinate lesion is produced, and as the margin advances, the process begins again in the center and similarly advances peripherally. With repetition concentric rings of white scales are formed. The lesions may be widespread over the body, those from different foci intermingling to produce a complex pattern. There is some disagreement as to whether the nails can be invaded.

Hyphae and spores may be found in the scales, and the appearance is very similar to that of Epidermophyton scales. Primary cultures must be made in liquid mediums. Growth on maltose agar is scanty and the colony a light gray with a central knob. On glucose agar growth is profuse; the colony is wrinkled and grayish white, changing to amber or brown. The growth consists largely of sterile hyphae and is not characteristic.

Diagnosis. As indicated earlier, classification of the dermatophytes is based largely on the clinical character of the infection and on colonial morphology on maltose-peptone agar. The differentiated structures developed in culture are, in some instances, of ancillary value. Fermentation reactions, emphasized by Castellani, are generally regarded as of little or no differential value. Animal inoculation is of minor importance unless the lesion is atypical and it is desirable to establish pathogenicity.

The probable cause of the disease can often be approximated on the basis of its clinical character; the usual associations are summarized in the accompanying outline. Demonstration of the fungus by direct microscopic examination, however, is essential to establish the etiology and culture is often required for species identification.

Association of Dermatophyte Species with Clinical Character of the Infection

(1) Lesions of the smooth skin:
  (a) Lesions of the moist epidermis of the principal folds:
    (i) Eczema marginatum, usually confined to the crural region:
      EPIDERMOPHYTON
    (ii) Dyshidrosiform intertriginous mycoses of the feet and sometimes hands:
      EPIDERMOPHYTON
      ectothrix TRICHOPHYTON
  (b) Circinate lesions on the backs of the hands, arms, face, neck, and trunk:

(i) Intricate pattern of concentric rings—tinea imbricata:
ectothrix TRICHOPHYTON
(ii) Reddish patches, not raised, with actively advancing margin and a tendency to central healing:
usually MICROSPORUM
sometimes endothrix TRICHOPHYTON
(iii) Reddish, elevated, scaly plaques, usually pustular at the margin:
endothrix TRICHOPHYTON
(2) Lesions of the hair and hair follicle:
(a) Scutula present, hairs tend to split longitudinally and not break off transversely:
ectothrix TRICHOPHYTON
(b) No scutula present, hairs tend to break off transversely:
(i) No suppuration, as follicular abscess, pustules, kerion:
1. Hairs broken off at uniform height several millimeters above the skin, scaling, spores in irregular clusters on the outside of hairs:
MICROSPORUM
2. Hairs usually broken off flush with the follicular orifice, little scaling, mycelium and chains of spores within the hair:
endothrix and neo-endothrix TRICHOPHYTON
(ii) Suppuration, lesions of smooth skin often present, fungus both in and on the hair, spores largely external in chains:
ectothrix TRICHOPHYTON
(3) Lesions of the nails, no distinctive clinical features:
TRICHOPHYTON
EPIDERMOPHYTON

The specimen material must be chosen with some care and taken in abundance for both microscopic examination and culture. In ringworm of the scalp epilated stumps of hairs may be taken, and in addition scutula should be taken in favus and the contents of abscesses when the infection is suppurative. In infections of the smooth skin, scrapings from the scaly types should be taken from the margins, rolling toward the normal skin, and the tops of vesicles may be clipped with small scissors in the vesicular types. Macerated epithelium may be taken from intertriginous infections and nail scrapings and subungual hyperkeratotic masses from onychomycosis; the fungous elements are often more difficult to demonstrate microscopically in such specimens.

For direct microscopic examination the material is placed on a slide, a few drops of strong (20 to 40 per cent) sodium hydroxide solution added, covered with a coverglass, and the preparation heated slightly. Only mycelial elements and spores may be found in specimens. Permanent stained preparations may be made by mounting in Amann's medium, a lactic acid-glycerol-phenol solution containing cotton blue. As indicated earlier, sections may be stained by Gram's method or with methyl green–pyronine.

Sabouraud's agar is the medium of choice for primary isolation as well as determination of colonial character. Inoculation should be relatively heavy and a number of plates prepared. Growth is

Figure 207. Dermatophyte colonies on Sabouraud's agar. *Left, Trichophyton mentagrophytes* var. *granulosum.* The colony shown is the granular type, almost all pure white except for a slight tan cast. *Right, Epidermophyton floccosum.* The colony is a light olive drab in color with a more deeply colored area in the center.

relatively slow, usually 10 days to 3 weeks are required, and the dermatophytes grow well at 30° C. Bacterial contamination may be a source of considerable difficulty, and it must be remembered that contamination with saprophytic fungi from air and dust readily occurs.

YEASTS AND YEAST-LIKE FUNGI

Yeasts are often defined as unicellular, nucleated organisms which reproduce by budding. Such a definition is generally recognized as inadequate, in part because some yeasts reproduce by fission, in part because many, possibly all, produce mycelium under appropriate conditions, and in part because other fungi may exist in a unicellular, yeast-like form which reproduces by budding, *viz.*, the oidia described in the previous section. On the basis of sexual spore formation, some yeasts are ascomycetes, others are probably basidiomycetes, and still others have not been shown to have a sexual stage and are grouped with the Fungi Imperfecti. Clearly, then, the term "yeast" is of somewhat uncertain significance; as commonly used it refers to those organisms which exist usually or predominantly in a yeast-like form.[80]

The yeasts are usually divided into three large groups, the basidiospore-forming yeasts or Sporobolomycetaceae, the ascospore-forming yeasts Endomycetaceae, and the asporogenous yeasts Torulopsidaceae. The industrial yeasts are, perhaps, the most familiar of these. *Saccharomyces cerevisiae*, a member of the second group, is the common brewing yeast and occurs as two types, top yeasts which produce a vigorous evolution of carbon dioxide and are found in the froth on the surface of the fermenting mixture, and the bottom yeasts which sink to the bottom. Bread yeasts are usually top strains of *S. cerevisiae*. Another species of this genus, *S. ellipsoideus*, is the common wine yeast, occurring naturally on grapes and in the soil of vineyards, and its varieties are named for the various types of wine which they produce. Still other yeasts are lactose-fermenters and are associated with the preparation of fermented milk beverages, such as kefir and koumiss, especially in southeastern Europe. Perhaps the commonest yeasts encountered as contamination in bacterial cultures and found growing on foods are the asporogenous torulae; the pink or coral pigmented forms often observed are *Torula glutinis*.

In view of the ubiquitous distribution

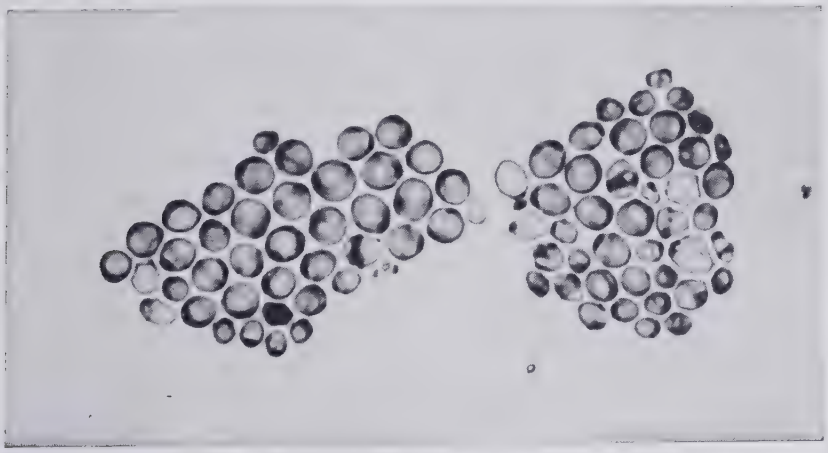


Figure 208. *Saccharomyces cerevisiae.* Smear from a pure culture. Methylene blue; × 1050.

Pathogenic Yeasts and Yeast-like Fungi

ORGANISM	DISEASE	GEOGRAPHICAL DISTRIBUTION
Cryptococcus neoformans	European blastomycosis, torula meningitis	Europe and U. S., probably ubiquitous
Blastomyces dermatitidis	American blastomycosis	U. S., possibly Europe and elsewhere
Candida albicans	Moniliasis	Ubiquitous
Coccidioides immitis	Coccidioidomycosis	Southwestern U. S. and South America
Histoplasma capsulatum	Histoplasmosis	Probably ubiquitous, most cases reported from South America and U. S.

of yeasts, not only in air, dust, and soil, but on the surface of the body and in the mouth, intestinal tract, and vagina, it is not surprising that these forms have been found in a variety of pathological processes. A great number of species have been described, most of them inadequately, in this connection. In many instances the yeast probably had no etiological relation to the disease, and in others the same yeast has been repeatedly described as a new species to give rise to several synonymous names, and a very long list of "pathogenic" yeasts has accumulated.

Critical examination and consideration has now made it clear that only a very few species of yeasts are actually pathogenic for man and animals. These fall into two groups, the one containing the causative organism of European blastomycosis or torula meningitis, commonly known as *Cryptococcus hominis* or *Torula histolytica.* The second group is made up of the "medical monilias," members of the subfamily Mycotoruloideae of the family Torulopsidaceae, which produce superficial infections of the skin and mucous membranes.

In addition to these a number of yeast-like fungi are considered in this section. These, together with the mold-like fungi discussed earlier, may be regarded as intermediate forms. The differentiation of these forms into "yeast-like molds" and "mold-like yeasts" implied by this separation is largely arbitrary.

CRYPTOCOCCUS NEOFORMANS (TORULA HISTOLYTICA)

In medical literature the term *blastomycosis* has been used very loosely to designate etiologically diverse infections in which budding cells are found in the tissues. Confusion arises largely between European blastomycosis, which will be considered here, and American blastomycosis, taken up in the following section.

Aside from moniliasis, two general types of yeast infection may be distinguished, the one the deep-seated cutaneous or subcutaneous infections which tend to become generalized, and the other infections of the central nervous system arising, as a rule, by metastasis from foci in the lungs. The first of these is known as European blastomycosis and the second at torula meningitis.

Cryptococcosis. What was probably the first case of yeast infection of proved etiology was a fatal generalized infection observed by Busse and by Buschke in 1893 which they called systemic blastomycosis and which subsequently has been generally known as European blastomycosis. From primary ulcers on the face and neck the infection spread to the cervical lymph nodes, and the causative organism was isolated first from a secondary tibial abscess, then from the primary ulcers, and shortly before death from the blood stream. Other cases reported since are of this general type, characterized by deep-seated ul-

Figure 209. Giant colony of *Torula glutinis*, a saprophytic species, on Sabouraud's agar; × 2.

ceration of the skin,[184] sometimes granulomatous, and there may be infection of the viscera, usually secondary, involving the spleen, liver, kidneys, and mesenteric lymphatics. Infection of the lungs may be primary, occasionally arrested with no further manifestations, or infection may spread from a focus in the lungs; lung infection may also be secondary.[76, 94]

The microorganisms are found in exudates and in mucoid masses of gelatinous material as round to oval cells, 5 to 6 μ in diameter, surrounded by a mucilaginous sheath. The gelatinous material in which they may be embedded is evidently a product of the fungus. It is reported that the cells may be stained specifically in tissue sections and smears by the fluorescent antibody technique.[91] They are readily cultivated on most ordinary mediums as a smooth white or very light tan colony without distinguishing features.

A number of cases of meningitis of yeast etiology have been reported, the great majority from this country. The disease is not common and less than 200 cases have been reported. It is almost always fatal in untreated cases[18] but amphotericin B therapy has been reported to be effective.[8, 168]

The clinical features of the disease set it off from European blastomycosis, for the infection is predominantly one of the central nervous system, and the skin is seldom involved. The symptoms are those of brain involvement, especially intracranial pressure. Brain tumor may be closely simulated in some cases, and the disease develops slowly, usually without febrile reaction or other signs of infection; a case of 16 years' duration has been reported. The pathological picture is that of chronic leptomeningitis with thickened meninges adherent to the cerebral cortex and showing diffuse or focal granulomatous lesions. The cerebral cortex is invaded in about half the cases; the lesions are sometimes granulomatous but more often cystic, and there is little if any inflammatory reaction. The granulomatous lesions of both meninges and brain contain large accumulations of macrophages which phagocytose the fungus, while the cystic lesions consist of enormous numbers of yeast cells embedded in a gelatinous matrix. The yeast is usually present in the spinal fluid in pure culture and may be observed in wet unstained preparations of the centrifuged sediment. The lung and kidney are the next most frequently involved, but any tissue may be attacked with generalization of the infection.[117, 124, 179] While there is as yet no definitive evidence, there is reason to believe that infection is more common than has been supposed, perhaps 2000 for every proven case.[143]

Infection occurs in lower animals, notably in the lungs and nasal granulomata of horses, and cryptococcus has been described as the etiological agent in a severe outbreak of bovine mastitis, but there is no reason to believe that lower animals constitute a reservoir of human infection. The organism has been isolated from soil,[3, 89] and it seems probable that this is the source of both human and animal infections. The microorganism is virulent for mice with an LD_{50} of about 1000 cells on intracerebral inoculation; following intraperitoneal inoculation it produces a generalized infection that spreads to the central nervous system. Experimental infection has also been produced in marmosets (*Leontocebus geoffroyi*) by feeding large numbers of the microorgan-

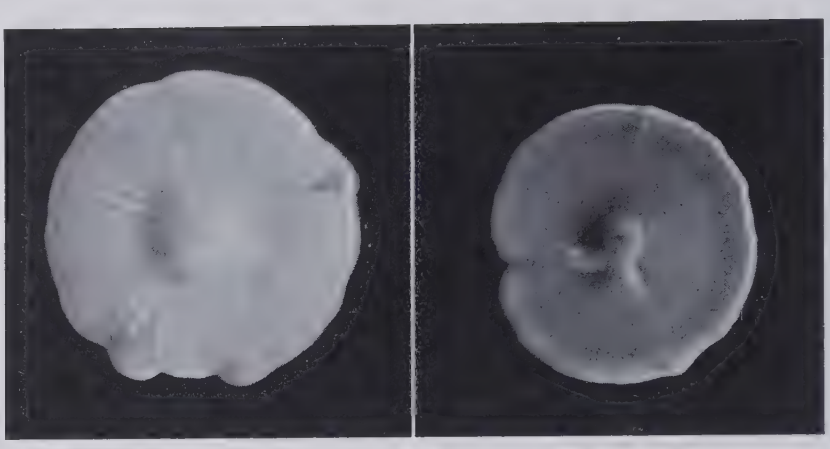


Figure 210. Giant colonies of torulae isolated from torula meningitis. Strain on left isolated by Shapiro and Neal, on right by Hirsch. Four weeks old, wort agar, natural size.

isms.[182] A minor degree of immunity, indicated by a reduced multiplication of the microorganisms, is demonstrable in experimental animals,[1] and infected animals give a delayed type of skin reaction to soluble cryptococcal antigen.[143]

The Causative Organism. For some time the relationship of these clinically diverse infections was not realized, and the yeast of European blastomycosis was known as *C. hominis* and that of torula meningitis as *T. histolytica.* In 1934, however, Benham showed that strains of the two yeasts were substantially identical, the difference in pathogenicity being one of degree only. Similar observations have been reported by others. It seems established, therefore, that these diseases are caused by the same etiological agent and, furthermore, that only a single species of yeast is involved, observed strain differences having no more than variant status. It characteristically ferments glucose, levulose, mannose, and sucrose slowly with the formation of acid and no gas, but this biochemical character is not distinctive.

This is *C. neoformans* (*C. hominis, Debaromyces neoformans, Torula histolytica*). A nonpathogenic variety, *C. neoformans* var. *innocuus,* and three other species, *C. laurentii, C. mucorugosus,* and *C. luteolus,* are accepted as valid species,[11] the last, however, has been found as the apparent etiological agent in pulmonary infection.[13]

The heavily encapsulated forms are poor immunizing antigens, but satisfactory antiserums may be prepared in the rabbit with poorly encapsulated forms. The microorganism gives a Quellung reaction in homologous antiserum. Evans and his co-workers[46, 47, 48] have shown that three immunological types occur, types A, B, and C, the type specificity depending upon the polysaccharide capsular antigen; these polysaccharides are closely related chemically, all containing xylose, mannose, galactose, and a uronic acid.

BLASTOMYCES DERMATITIDIS (NORTH AMERICAN BLASTOMYCOSIS)

The fungous disease known as North American blastomycosis was first observed in Baltimore in 1894 by Gilchrist and is sometimes called Gilchrist's disease. The great majority of cases have been found in this country[149, 150] and are concentrated in the north central and eastern states,[151] hence the name American blastomycosis, and the disease has been observed more often in the state of Illinois than in other states and is occasionally called Chicago disease. According to Martin and Smith[108] only two proved cases have originated outside the United States, one in Canada and one in

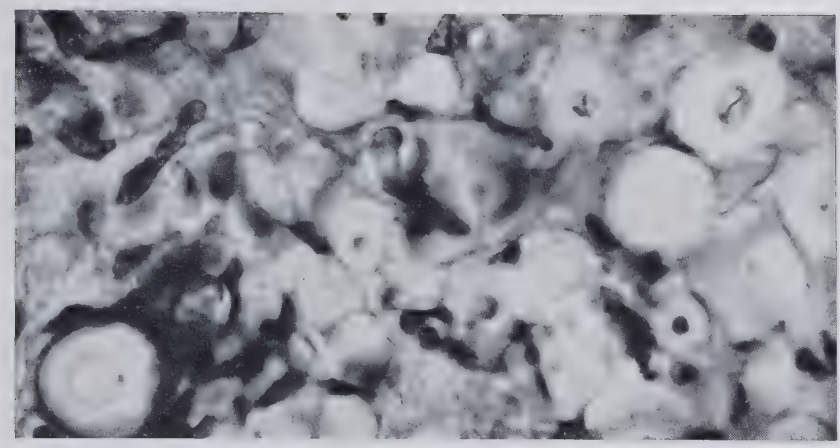


Figure 211. Torula meningitis in man. Section of the brain showing the growth of *Cryptococcus neoformans* in the tissues. Note the yeast-like cells embedded in masses of hyaline material which fill and distort the macrophages. Hematoxylin and eosin; × 950. (Humphreys.)

England, though analysis of presumptive and inadequately described cases is difficult because of the usual failure to distinguish this disease from European blastomycosis.

The Causative Organism. This fungus was isolated in 1896 by Gilchrist and Stokes from a second case of the disease and named *Blastomyces dermatitidis.* It is dimorphic, occurring only as a unicellular, budding, yeast-like form in the tissues, but as a mycelial form in culture. The unicellular form may be observed in sodium hydroxide preparations of pus or sputum. The cells are large, 8 to 10 μ in diameter, round or oval, and doubly contoured. The granular content of the cells distinguishes them from air bubbles or fat droplets, and identification is practically certain if budding cells are found.

Colonial morphology. This unicellular morphology also occurs in cultures on blood agar, microscopic examination showing budding cells with a few rudimentary hyphae. The colony developing at 37° C. is wrinkled and waxy and somewhat similar to those of the tubercle bacillus in appearance. In culture on Sabouraud's agar the mycelial form appears. The colonial morphology is somewhat variable, and three types are distinguished. The "mealy" type is most often observed in primary isolation cultures and is similar to the growth on blood agar; microscopically the fungus is transitional between the unicellular and mycelial forms with many of the cells tending to form articulated chains. After one or two transfers the growth may assume a prickly surface, the prickles consisting of closely packed mycelial filaments which tend to become loose and cottony with continued incubation. The third type, sometimes observed on primary isolation and commonly found on laboratory cultures which are frequently transferred, is characterized by a white cottony growth with abundant aerial mycelium; in these colonies the unicellular form has completely disappeared, and the growth consists of septate hyphae. Conidia are borne on lateral conidiophores, but reports by some workers of the formation of asci have not been generally confirmed. The mycelial form reverts to the unicellular type in experimental infections or when cultivated on blood agar.

Classification. The relationships of this fungus are somewhat uncertain though it is clearly not a "true yeast." Taxonomic considerations have led to the proposal of a number of different names; these include *Oidium dermatitidis, Gilchristia dermatitidis, Cryptococcus gilchristi, Mycoderma dermatitidis, Zymonema dermatitidis,* and others. Systematists object to all these for one reason or another, and none has been generally accepted. In spite of the fact that, strictly speaking, the generic name

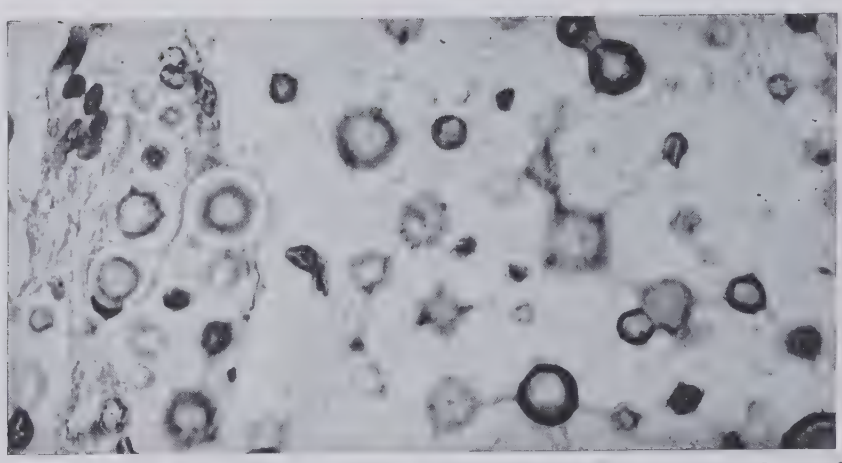


Figure 212. Torula meningitis in man. Growth of *Cryptococcus neoformans* in the cerebrospinal fluid. Note the budding cells, the stained capsules which give a double-contoured appearance, and the characteristic threads connecting the cells. Mucicarmine stain (staining the threads and capsules pink); × 950. (Humphreys.)

Blastomyces is improper from the taxonomic point of view, it continues to be generally used and the fungus is known as *B. dermatitidis.*

Pathogenicity.[10, 19, 169] The primary lesion occurs most commonly in the skin, and the lesion begins as a small firm papule about which secondary nodules develop which enlarge with coalescence. The process breaks down in the center and becomes suppurative with discharge of pus through small fistulas. The inflammatory reaction is granulomatous with connective tissue formation, proliferation of the epithelium, mononuclear infiltration, and sometimes giant cell formation. The fungus is found in the pus of minute miliary abscesses which are a characteristic feature of the pathology. As the disease progresses, therefore, a large elevated mass of tissue, with an irregular ulcerated surface, is formed and oozes pus from multiple small openings upon pressure. The resemblance to epithelioma or tuberculous ulcer is sometimes striking. Lesions with pronounced epithelial proliferation simulate tuberculosis verrucosa. The process spreads slowly through the subcutaneous tissues and often becomes generalized by way of the blood stream.

Primary infection of the lungs is not uncommon and often closely resembles tuberculosis clinically with cough, pain in the chest, weakness, sometimes hemoptysis, and productive sputum late in the disease. The pathological picture is variable in that there may be focal or diffuse consolidation, and the abscesses may be miliary or there may be larger nodules. Cavitation occurs but is limited to small areas. The microscopic picture also resembles tuberculosis, and sometimes it is difficult to differentiate unless the budding cells are found.

With generalization from primary foci of infection, multiple small abscesses of hematogenous origin occur throughout the body. They are most common in the subcutaneous tissues and differ from the primary skin lesions in that they develop without pain or marked erythema, are soft, and evacuate considerable quantities of pus when opened. In contrast to the primary skin lesions, which most commonly occur on exposed parts of the body, the secondary subcutaneous abscesses are usually found in covered areas. Secondary abscesses also commonly develop in the viscera and muscles and under the periosteum. The disease may be chronic and persist for years, but in the generalized infection there is a septic febrile reaction and the case fatality rate is high. The diamidines, stilbamidine and 2-hydroxystilbamidine, have therapeutic activity,[70, 99, 175] but relapses are not un-

common. More recently, amphotericin B has given very promising results.[71, 167]

The source of infection is not clear. Very often the primary skin lesion develops from a wound infection and a large proportion of the cases have been in farmers. There is reason to believe that in many cases pulmonary infection is primary, presumably acquired by inhalation, and in some cases there are no other lesions, or cutaneous lesions may be overtly primary but secondary to the lung infection. Males are much more frequently attacked, and the incidence is higher in the 20- to 50-age group. It is possible that the fungus is a free-living form, for it has been recovered from soil,[33, 110] which, like Sporotrichum, occasionally infects man. One case of direct transmission, by autopsy infection, is known.

Pathogenicity for animals. The inoculation of experimental animals is not uniformly successful and has no diagnostic utility. Mice are more susceptible than guinea pigs, and rabbits are almost completely resistant. Small caseous nodules develop on the peritoneal surfaces of intraperitoneally inoculated animals, and the type of tissue reaction varies with the resistance of the animal and the virulence of the strain of fungus from frank abscess formation to tubercle-like lesions. An instance of canine infection apparently transmitted to man has been reported.[148]

Immunity. There appears to be little or no effective immunity in man to spread of the infection since generalization is common. Complement-fixing antibodies are produced, however, and the titer is related to the severity of the infection.[106] Hypersensitivity to the cell substance of the fungus occurs also and is manifested as a delayed tuberculin-like response to intradermal inoculation of suspensions of killed cells. The hypersensitivity is of practical importance in that it contraindicates the therapeutic use of iodides; desensitization may be accomplished with vaccine. It has not as yet been possible to demonstrate an effective immunity to the experimental infection in laboratory animals.

Diagnosis.[107] As indicated above, the yeast-like unicellular form of the fungus can be demonstrated by direct microscopic examination of pus or sputum mounted in sodium hydroxide. Positive complement-fixation and skin tests are of value in pulmonary and systemic infections. An unequivocal diagnosis can be established, however, only by isolation and identification of *B. dermatitidis.* This is usually not difficult if the specimen is not too heavily contaminated with bacteria; the fungus grows readily on Sabouraud's agar, the

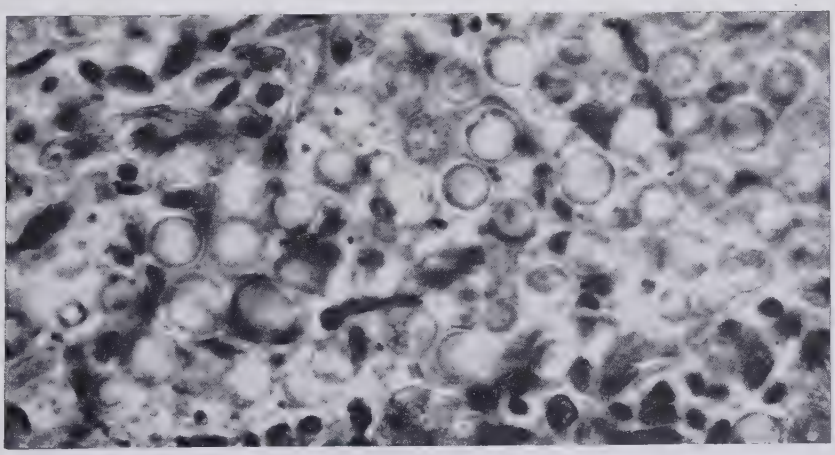


Figure 213. Blastomycotic pneumonia in man; infection with *Bastomyces dermatitidis.* Note the enormous numbers of yeast cells in the lung tissue and the variation in size and budding forms characteristic of active proliferation. Hematoxylin and eosin; × 1950. (Humphreys.)

colonial morphology varying as indicated earlier.

CANDIDA ALBICANS[102] (MONILIA ALBICANS)

The fungi which makes up the group of "medical monilias" are yeast-like organisms which are usually found in the budding unicellular stage. Mycelium is formed only under semi-anaerobic conditions or in submerged cultures and usually is found in the tissues. When cultured on agar in the usual way, the colonies are smooth and waxy and on microscopic examination are found to consist entirely of budding, yeast-like cells. Gelatin stab culture is a convenient method of differentiating these forms from the yeasts for, while the surface growth is yeast-like, growth along the line of inoculation includes the extension of radiating filaments into the medium; these consist of mycelium from which budding cells arise. The same type of growth may be observed on agar plates by cutting deeply into the medium with the inoculating needle and examining the growth through the bottom of the plate with the low power. In general, under conditions favorable for growth and rapid multiplication, the yeast-like form predominates, while mycelium appears under less favorable conditions.

The first description of a fungus of this type occurring in disease was that of Langenbeck in 1839 who found it in patches on the mucous membranes of the mouth and elsewhere at autopsy. The organism was named *Oidium albicans* by Robin 4 years later. The generic name Candida has come to be commonly used, and the disease is usually termed *candidiasis.* The diseases are usually superficial infections of the mucous membranes, the intertriginous areas, and the nails, but pulmonary candidiasis is known, and localized infections sometimes result in a generalized infection by hematogenous spread, especially in hosts debilitated by some other disease.

The Causative Organism.[158] In addition to the original *Candida albicans,* a considerable number of species of Candida have been described including *C. krusei, C. parakrusei, C. tropicalis, C. stellatoidea,* and others. In fact, a large proportion of current literature on these organisms is devoted to their differentiation, identification, and classification.[133] Differentiation is made on the basis of various cultural characters such as pellicle formation in liquid mediums, gelatin liquefaction, and the like, and Castellani emphasized differential fermentations. The last is the most practical laboratory method, but there is some disagreement as to the constancy of these characteristics.

These various strains are immunologi-

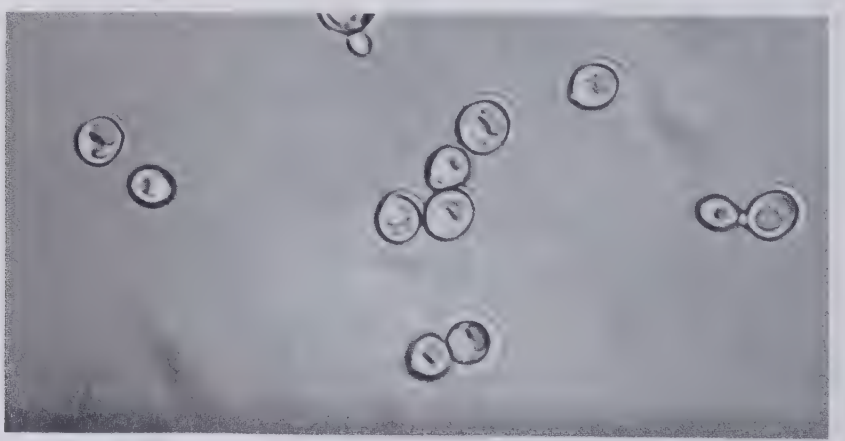


Figure 214. Unstained preparation of the yeast-like form of *Candida albicans.* Oblique lighting. Note the budding cells and close resemblance to the true yeasts. × 1050. (Rothman.)

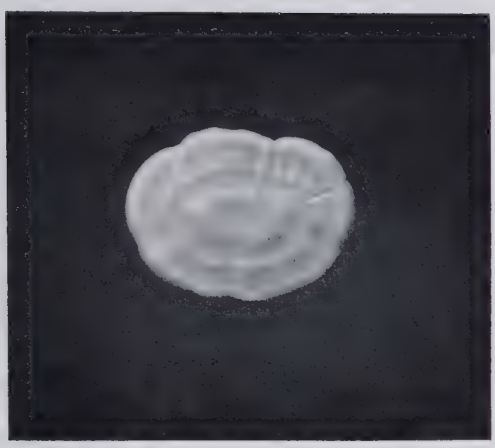


Figure 215. Giant colony of *Candida albicans* on Sabouraud's agar. Note the smooth creamy growth characteristic of yeasts. × 3.

cally related and show marked cross reactions, but the group is not homogeneous. A detailed antigenic analysis carried out by Tsuchiya and his co-workers[185] has shown the essential identity of *C. albicans* and *C. tropicalis,* and the separation of *C. stellatoidea.* Similar studies by Hasenclever and his associates[73, 74, 75] with *C. albicans,* for which chlamydospore production is the usual criterion for identification, showed that it is separable into two antigenic types, designated groups A and B. *C. tropicalis* falls into group A, and *C. stellatoidea* into group B. Strains of *C. tropicalis* and *C. stellatoidea,* as well as *C. albicans* strains of both groups, are virulent for the mouse, but the virulence of *C. albicans* for the rabbit appears to be significantly greater than that of the other species.

Pathogenicity. These fungi are commonly present in the mouth, vagina, and intestinal tract in normal persons and are probably not highly virulent with respect to initiation of the infectious process. In fact, as in many of the mycoses, there is frequently a history of a debilitated condition or other predisposing factors. While in some instances in which they are associated with the development of a pathological process there is some doubt as to their etiological role, in many others it is clearly evident that they are primary invaders and responsible etiological agents.

There is evidence that treatment of other infections with the broad spectrum antibiotics, especially the tetracyclines and chloramphenical, increases the number of persons in whom Candida is found in the intestinal tract and vagina, and it is related to the incidence of candidiasis which occurs as a sequel to prolonged therapy with these agents. While the suppression of susceptible microflora antagonistic to the fungi is probably concerned in the overgrowth of Candida, it has been suggested that such antibiotics may, in fact, enhance the virulence of these fungi. There is a certain amount of experimental evidence supporting this[86, 87, 88] but its relative importance remains obscure.

Infections of the mucous membranes. Candidiasis of the mucous membranes is known as thrush and is one of the common mycoses. Intra-oral thrush, a superficial infection of the mucous membranes of the mouth, is the type most frequently observed and is considerably more common in nursing infants and children than in adults. In the past it has occurred in severe epidemic form in institutions such as foundling asylums, but such epidemics are observed less often now. Thrush occurs more frequently in bottle-fed than in breast-fed babies, and the lesions not infrequently spread to the pharynx and even the esophagus. In adults thrush generally occurs terminal to wasting diseases such as tuberculosis and cancer; dryness of the mouth associated with prolonged coma or near-coma appears to favor infection. It also occurs with some frequency as a mild vaginal infection in pregnant women which may or may not be associated with infection of the anus.

In the majority of cases the infection is mild and remains localized. Occasionally it may spread to other mucous membranes and to the skin with the development of a generalized cutaneous eruption[187] and intertriginous lesions, and such cases are sometimes fatal. In the cutaneous form in infants it is to be distinguished from infantile eczema and allergic hypersensitivities.[138] The most common predisposing malady for cuta-

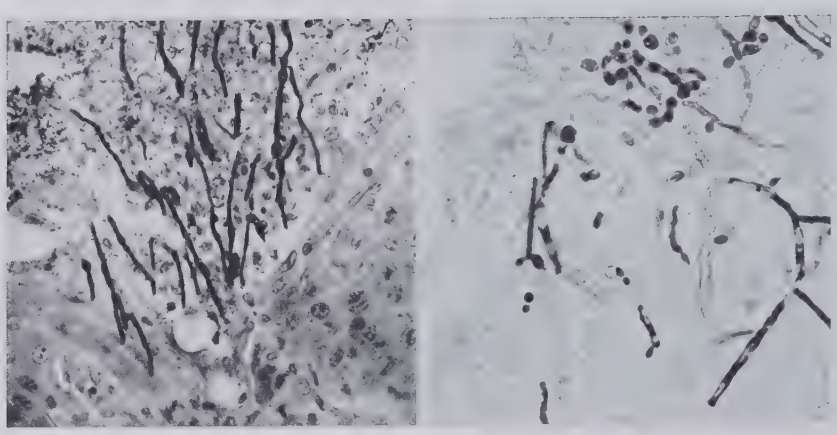


Figure 216. Candidiasis: invasion of the epithelial layer of the esophagus by *Candida albicans*. *Left*, beginning invasion of the superficial layer showing mycelium and yeast-like cells; note the gram-positive micrococci accompanying the fungus. × 600. *Right*, invasion of the deeper tissue showing both mycelium and yeast-like cells penetrating the epithelium. × 950. Gram stains. (Humphreys.)

neous candidiasis is diabetes. Hematogenous spread with metastasis and abscess formation in the viscera has been observed.

The lesions appear as soft, whitish patches, composed largely of fungous growth. They are readily removed to leave an eroded surface. There is some resemblance to a diphtheritic membrane, and lesions in the throat and on the tonsils have no doubt been mistaken for diphtheria. However, it is much easier to separate the membrane in candidiasis. On microscopic examination of the membranous material in wet preparation, the observation of a tangled mass of segmented mycelium admixed with budding yeast-like cells, desquamated epithelium, and leucocytes establishes the diagnosis. The fungus may be cultivated on Sabouraud's agar, but isolation in pure culture is more readily accomplished with dextrose tartaric acid agar. The isolated fungus must be differentiated from yeasts.

Dermatomoniliasis. *C. albicans* is also causally associated with eczema-like lesions of the moist skin similar to those produced by *E. floccosum*. Infection of the folds between the fingers, *erosio interdigitalis*, is more common than dermatophytosis of this region, and is found most often in those whose hands are frequently wet. *Perlèche*, an infection of the angles of the mouth, is another form of intertrigo produced by these fungi. *Moniliids*, sterile vesicular or exudative lesions which appear on the hands secondary to a focus of infection elsewhere, are analogous to trichophytids and a result of hypersensitivity.

The nails, chiefly of the fingers, are also attacked by *C. albicans* with the production of chronic paronychia. The condition is differentiated clinically from onychomycosis by retention of the luster of the infected nail and the absence of yellowish discoloration, crumbling, and thickening of the soft tissues. The nail shows transverse ridges and eventually becomes thickened, distorted, and brownish in color. Like interdigital infections, the infection is found most often in those whose hands are often wet.

On microscopic examination of nail and skin scrapings cleared in 10 per cent sodium hydroxide, hyphae may be present together with the budding yeast-like cells.

Bronchomoniliasis.[22] There appears to be a distinct type of candidiasis of the respiratory tract which was first reported by Castellani from Ceylon. It has been observed in Europe and in this country but is apparently more common in the tropics; its frequency as a primary infection is uncertain. According to Castellani, two types of the disease occur; the mild form, chronic bronchitis characterized by dyspnea and cough, is afebrile, while the

severe form is similar to tuberculosis and usually fatal.

The fungi may be demonstrated by direct microscopic examination and by culture, but such findings must be interpreted with caution,[9] for the organism is frequently found in the sputum in other diseases, especially tuberculosis. It is recommended that the throat be cleansed by gargling before the sputum is collected and other contamination avoided. Strains isolated from cases of bronchomoniliasis are pathogenic for rabbits (see above), producing tubercle-like nodules within 2 or 3 weeks after inoculation into the lungs.[57]

Figure 217. Colony of *Coccidioides immitis* on blood agar. Isolated from a fatal case of coccidioidomycosis, natural size.

COCCIDIOIDES IMMITIS[50]

Coccidiomycosis, coccidioidomycosis, or coccidioidal granuloma was first observed in Argentina in 1892 by Posados and by Wernicke; 2 years later it was described independently in California by Rixford. The causative organism was thought to be a protozoan and named *Coccidioides immitis*. Ophuls and Moffitt showed, by culture, that it is a fungus, but this does not invalidate the name. Though originally reported from South America, the disease appears to be rare and is most prevalent in the San Joaquin Valley in California and in the dry regions of the southwestern United States; it seems to be unknown elsewhere.

The Causative Organism. Like a number of the other pathogenic fungi, *C. immitis* is dimorphic, and the parasitic stage within the tissues is morphologically different from that assumed in culture.

The parasitic stage. This fungus differs markedly from the yeast-like fungi in that it never reproduces by budding, and it differs from all the other pathogenic fungi in that it reproduces within the tissues exclusively by a process of endogenous spore formation. The newly liberated spores are small mononucleate spheres 1 to 3 μ in diameter and appear as a central, deeply stained mass of protoplasm surrounded by a double-contoured capsule. The cell enlarges, soon becoming multinucleate, and eventually reaches a diameter of 50 to 60 μ. A central vacuole appears early and in later stages occupies a large portion of the cell, the protoplasm appearing as a thin peripheral layer. The peripheral protoplasm becomes vacuolated and an indefinite number of multinucleate protospores become delimited by the formation of cell walls; the protospores in turn subdivide to form spores and the entire cell assumes the functions of a sporangium. With rupture of the cell wall the mature spores are liberated, and the developmental cycle begins again.[39] All of these forms may be observed in the tissues and in pus, though recently disseminated spores are difficult to demonstrate. Hyphal forms have been observed in the tissues but are very rare.[130] The morphological phase found in the tissues is determined by various environmental factors.[26, 100]

The saprophytic stage. The fungus will grow on a variety of sugar-containing mediums but Sabouraud's agar is the medium of choice. It grows best at 37° C. under aerobic conditions. On artificial culture mediums the growth is that of a mold. The colony may be smooth and waxy when young, aerial mycelium is soon formed and it becomes gray or brownish in color. Emmons has pointed out that, though it is often stated that the aerial hyphae break up into chlam-

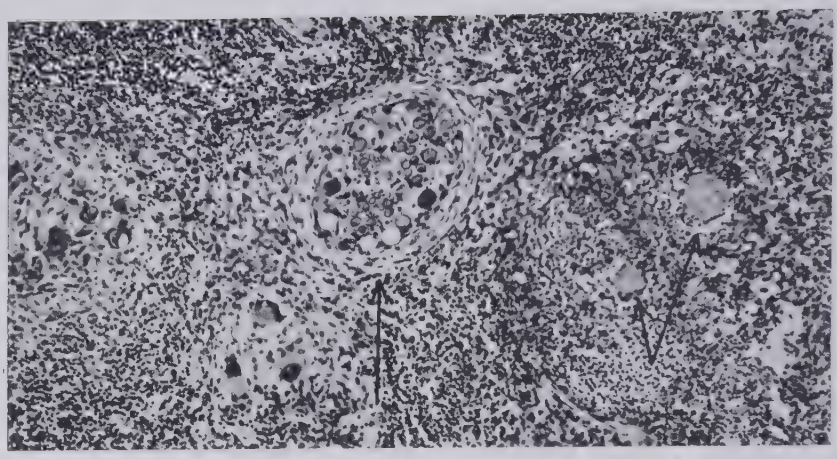


Figure 218. Coccidioidomycosis in man. Section of spleen showing a large lesion of the chronic type and two smaller lesions characteristic of the acute type of infection. In the latter sporulation is complete and the spores are beginning to spread into the surrounding tissue. Hematoxylin and eosin; × 125. (Humphreys.)

ydospores, the spores are borne on differentiated conidiophores and should be termed conidia or oidia. Spirals are frequently observed.

Pathogenicity.[85, 166] For many years infection with *C. immitis* was known only as coccidioidal granuloma, a severe disease in which the case fatality rate was thought to be 90 per cent or more. Infection with this fungus is, however, much more prevalent and less severe than had been supposed. Healed lesions of coccidioidal granuloma are found at autopsy of persons dying of other causes, and similar arrested lesions have been produced in white rats. Furthermore, it was shown by Dickson in 1937 that the disease may take a second form, a benign, acute, self-limited respiratory infection, in addition to the chronic, progressive granulomatous disease. He suggested that these be designated primary and secondary or progressive coccidioidomycosis respectively, and these terms are now coming into general use. In South America the infection is known as Posada's disease.

Primary coccidioidomycosis.[128] The infection is acquired by inhalation of the spores, and the disease varies in severity in recognized cases from that of a common cold to cases resembling influenza with pneumonia, cavitation, and high fever. In a small proportion of the cases, possibly 5 per cent, an exanthem-like erythema nodosum occurs, and the disease is recognized as "the bumps," desert rheumatism, valley fever, San Joaquin fever, etc. Many infections are symptomless, however. In a group of 1351 cases studied by Smith et al.[165] 60 per cent were symptomless, and dissemination with development of the progressive disease occurred in about 1 per cent of the clinically apparent infections. They also noted that dissemination occurred ten times as frequently in the Negro as in the white. Minor "epidemics" may occur such as that described by Davis, Smith, and Smith,[32] in which seven of fourteen persons who spent 2 days on a desert field trip near the San Joaquin Valley became infected, and Goldstein and Louie[64] reported that of several thousand soldiers exposed during military training in a desert endemic area, seventy-five contracted the disease. All but one made rapid progress to complete recovery, but the remaining individual developed a generalized infection and coccidioidal granuloma. Spontaneous recovery is the rule, and only rarely does the disease progress to the secondary type. Residual pulmonary lesions, closely resembling healed tuberculous lesions, are common.[4, 81, 173]

Secondary or progressive coccidioidomycosis. The progressive type of in-

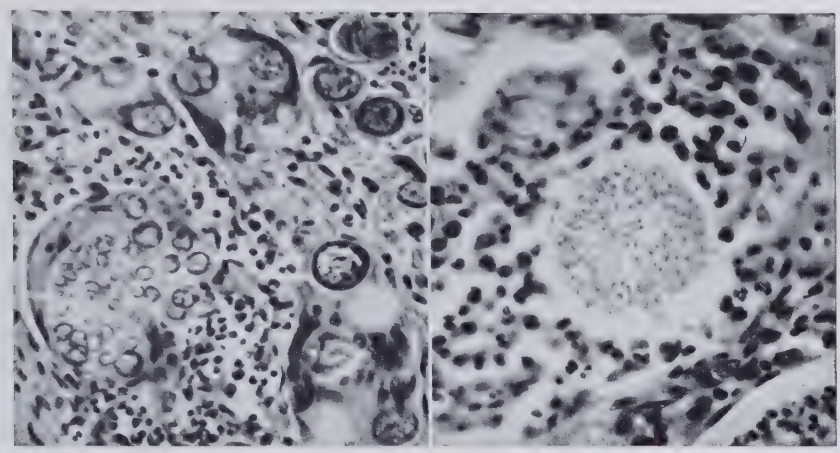


Figure 219. The chronic and acute types of lesion in coccidioidomycosis in the same section shown in Figure 218, but under higher magnification. *Left*, the chronic type of lesion showing many mature cells of *Coccidioides immitis*. × 475. *Right*, the active type of lesion showing a mass of spores beginning to spread in the tissue. Hematoxylin and eosin; × 710. (Humphreys.)

fection results in cutaneous, subcutaneous, visceral, and osseous lesions. The cutaneous type closely resembles blastomycosis but is a much more severe disease with fever and a greater tendency to hematogenous spread. Elsewhere the lesions closely resemble those of tuberculosis, and, in fact, differentiation from that disease may often be possible only by demonstration of the fungus. It is not known whether the progressive type of disease results from a new infection or reactivation of arrested primary lesions.[163] Amphotericin B is reported to be an effective chemotherapeutic agent in the disseminated infection.[23, 84]

Pathogenicity for animals. The disease occurs naturally in domestic animals including cattle,[103] sheep, and dogs and also in certain wild rodents in endemic areas. The latter include three species of pocket mice, *Perognathus baileyi, P. penicillatus,* and *P. intermedius,* the kangaroo rat, *Dipodomys merriami,* and the grasshopper mouse, *Onychomys torridus.* Experimental animals are readily infected; both rabbits and guinea pigs are susceptible to intraperitoneal inoculation and mice to intracerebral inoculation. Mice, inoculated intracerebrally[90] or intraperitoneally,[55] are used for virulence assay. The fungus is also cultivable in the embryonated hen's egg,[16, 139, 188] usually in the yolk sac.

Immunity. Since it is now apparent that infection with *C. immitis* can take a mild form from which spontaneous recovery is the rule, that arrested lesions occur with some frequency, and that the case fatality rates in the severe progressive type of disease are not as high as once thought, it is indicated that there is, on the one hand, an appreciable natural resistance to infection, and on the other, an effective immune response.

The immune response is manifested in part as the development of a hypersensitivity to the parasite. *Coccidioidin*[131] is, like tuberculin (OT), prepared from liquid cultures of the parasite, but there is some difficulty in reproducing potencies. An immunologically active polysaccharide appears to make up at least a part of the active principle, producing both skin reactions and precipitin test. Coccidioidin is inoculated intradermally, a positive reaction appearing in 24 to 48 hours. The hypersensitivity appears in a few days to a few weeks after infection, recent infections and severe infections resulting in stronger reactions than old infections and mild infections respectively. The test appears to be highly specific though there is as yet some disagreement as to its interpretation.

Humoral antibodies are formed also, and complement-fixation[164] and precipitin tests, using an autolysate of culture as the antigen, have been developed that

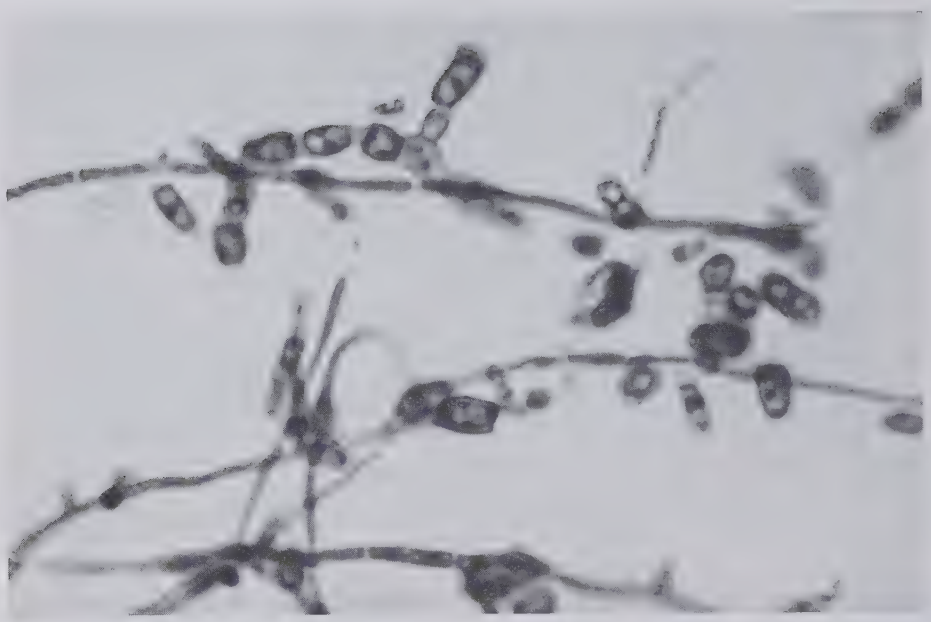


Figure 220. Arthrospores of *Coccidioides immitis*. Lactophenol–cotton blue preparation from culture on Sabouraud's medium; × 1135. (Havens.)

are specific. In general, serum antibody is not detectable prior to the development of skin sensitivity. Precipitin appears first and is demonstrable in 50 per cent of cases by the end of the first week of the disease, and in 90 per cent or more by the third week, and then gradually falls until no titer is demonstrable after 7 months. The complement-fixation reaction becomes positive more slowly, with about 8 per cent positives by the end of the first week, and reversion apparent by the second month. In general, then, precipitating antibody characterizes the primary stage of the disease with about 78 per cent positive reactions as compared with 55 per cent positive complement fixation reactions, while complement-fixing antibody occurs in the secondary or progressive stage in 98 per cent of cases in contrast with 36 per cent positive precipitin reactions. Thus the titer of complement-fixing antibody has considerable prognostic significance; rising titer is indicative of the development of the progressive granulomatous form of the disease, often before this is evident clinically.[105]

Diagnosis. The laboratory diagnosis of coccidioidomycosis is dependent upon the demonstration of the parasite, the diagnostic value of a positive coccidioidin reaction being somewhat uncertain. As indicated earlier, it may be found on direct microscopic examination of pus, spinal fluid, and tissues but may be difficult to demonstrate in the sputum. Lactophenol–cotton blue or Mallory's eosin and methylene blue may be used for staining. Though *C. immitis* grows without difficulty on sugar-containing mediums, it has been cultured in a surprisingly small proportion of cases. There is a very considerable hazard in culturing the fungus owing to the infectivity of the spores, and special precautions are required.[28]

Epidemiology. In the endemic areas of the southwestern United States the proportion of reactors to the coccidioidin test is high, ranging from 16 to 90 per cent. The prevalence of infection in these regions appears, therefore, to be much higher than suspected earlier. A very large proportion of these reactors have no history of coccidioidomycosis—Smith[50] estimated that only 5 per cent of infected persons show sufficiently pronounced clinical symptoms to be de-

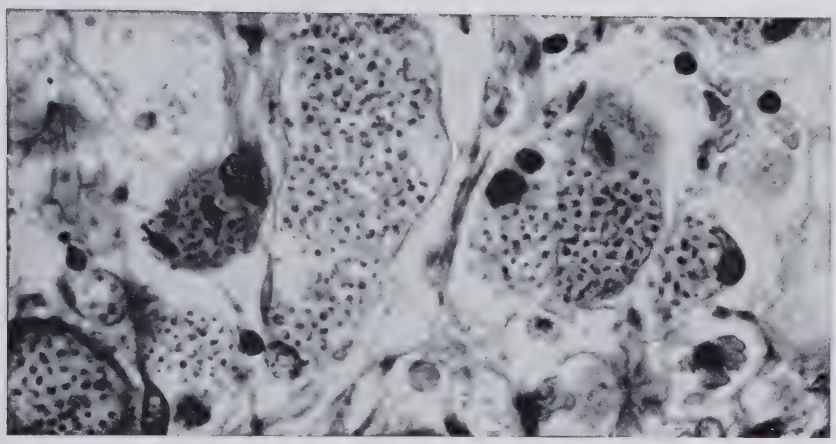


Figure 221. *Histoplasma capsulatum.* Yeast-like cells in the macrophages of the bone marrow in systemic histoplasmosis. Hematoxylin and eosin; × 1000. (Humphreys.)

tected and diagnosed. The disease is also readily acquired from cultures of the fungus, and a considerable number of laboratory infections have been reported.

Though, as indicated earlier, the disease occurs in domestic animals and certain wild rodents, there is no evidence of direct transmission of the infection from animals to man. It seems likely both man and animals are infected by the inhalation of spores contained in dust; there is frequently a history of exposure to dust storms in human cases, and the fungus has been isolated directly from dust. *C. immitis* is probably a saprophyte which infects animals and man by chance, the reservoir of infection of which appears to be the soil.[37]

PARACOCCIDIOIDOMYCOSIS

The disease known as paracoccidioidal granuloma, Lutz's disease, or South American blastomycosis occurs in Brazil and elsewhere in South America but has not been observed in this country. The causative organism is *Paracoccidioides brasiliensis* which, despite its name, is not closely related to *C. immitis* but is related to *B. dermatitidis*,[52, 53] and is also known as *Blastomyces brasiliensis.* It is a yeast-like fungus, proliferating by budding in the tissues, and grows in a compact cerebriform colony on Sabouraud's agar, eventually producing a white aerial mycelium.

The disease is clinically similar to coccidioidomycosis and blastomycosis.[101] The fungus enters the body via the mouth to produce ulcerative granulomatous lesions which spread to the tongue, lips, and nose. The infection spreads via the lymphatics to produce lesions of the viscera and often pulmonary involvement. It is usually fatal. It may also be acquired by inhalation. Guinea pigs are usually infected by intratesticular inoculation and may also be infected by intratracheal inoculation. The fungus will grow readily on the chorioallantoic membrane of the developing hen's egg.

Diagnosis is established by microscopic demonstration of the multiple budding cells (in contrast to the singly budding cells of *B. dermatitidis*) in material from the lesions or by culture.

HISTOPLASMA CAPSULATUM[122, 178]

This organism was discovered in 1906 by Darling who observed it in sections of tissues taken post mortem from three cases of what appeared to be visceral leishmaniasis and named it *Histoplasma capsulatum.* The first case in the United States was reported in 1926. The actual number of infections is undoubtedly many more than is indicated by reports of cases. Healed lesions of pulmonary

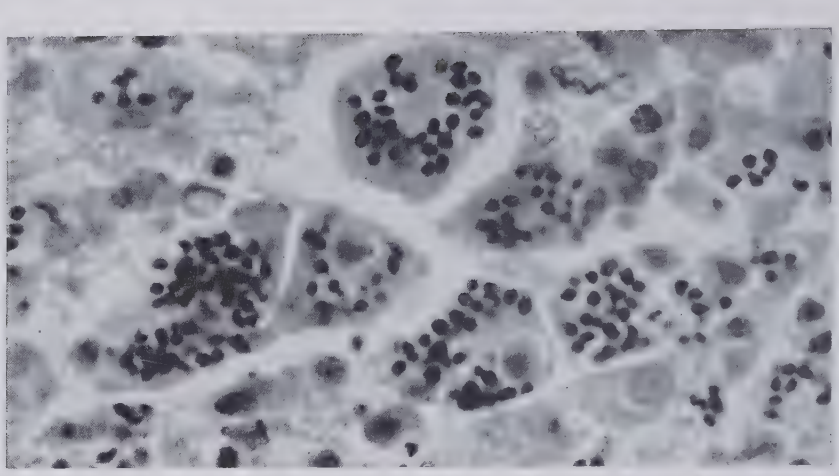


Figure 222. *Histoplasma capsulatum.* Yeast-like cells in the suprarenal gland in systemic histoplasmosis. Gram stain; × 1350. (Humphreys.)

infections are frequently found at autopsy in areas where the disease is prevalent,[147, 172] and the prevalence of the infection is shown by skin testing for hypersensitivity to the substance of the microorganism. The disease has been studied most intensively in this country, but apparently its distribution is worldwide.[36]

The Causative Organism. The organism appears in the tissues as a small, oval, encapsulated yeast-like cell 3 to 5 μ in diameter. Larger strains, up to 15 μ in diameter, are found in African histoplasmosis[35] and have been observed on occasion in this country. In stained preparations the central stained mass is surrounded by a clear zone. The organism is usually intracellular, in the mononuclears in the peripheral blood and in the macrophages elsewhere, especially those of the bone marrow and spleen. Because of this characteristic intracellular position of the parasite, the disease is sometimes called reticuloendothelial cytomycosis or simply cytomycosis.

The microorganism is cultivable from the blood or biopsy specimens, or it may be isolated from the sputum in pulmonary infections by animal inoculation, during the course of the disease, and from various organs and tissues at autopsy in fatal cases. It grows in the yeast-like form seen in the tissues in sealed blood agar cultures and in tissue culture,[96] but on Sabouraud's agar a mycelial form is assumed and the colonies are mold-like, white, and cottony with aerial mycelium. Chlamydospores are formed in abundance, first smooth and pyriform, but as they mature they become larger, 7 to 15 μ in length, thick-walled, and tuberculate with finger-like protuberances sometimes as much as 6 μ long. Conant[24] has studied these developmental stages in detail and has been able to convert the mycelial stage to the yeast-like form by cultural methods. The fungus has been regarded by some as closely related to *P. brasiliensis.*

Pathogenicity.[20] Four general types of disease produced by *H. capsulatum* are usually differentiated. The clinical picture is sometimes one of a systemic febrile disease with splenomegaly, septic temperature curve, anemia, and leucopenia, simulating kala-azar. A second type is distinguished by the predominance of lymphatic involvement and resembles Hodgkin's disease, lymphosarcoma, leukemia, or aplastic anemia. Or pulmonary symptoms may predominate with cavitation and fibrous adhesions of the pleurae, the infection sometimes complicating tuberculosis; the development of calcified intrathoracic nodules is perhaps a consequence of pulmonary infection. A fourth type is characterized by ulcerative skin lesions; ulceration also occurs in the intestine.

Though it is sometimes implied that histoplasmosis is always a generalized infection of the macrophage system, the parasites being especially numerous in tissues rich in these cells such as the spleen and bone marrow, this is by no means generally true. In most cases there are nodules or extensive areas of necrosis in one or more organs, but in some instances only a single organ, such as the adrenals, has been infected. These necrotic lesions usually consist of a central area of necrosis surrounded by granulation tissue containing large numbers of macrophages and ingested parasites. In some instances the parasite has been found to be limited to such areas, while in others it is widely distributed in the macrophage system as well. Infection of the central nervous system is relatively rare but has been observed.[145, 156] The statistical distribution of the lesions of the disease has been summarized by Schulz.[146]

Experimentally infected animals give a positive reaction to the intradermal inoculation of a preparation of liquid culture of *H. capsulatum* analogous to old tuberculin and coccidioidin and designated *histoplasmin.* In areas in which the disease is endemic, positive reactions are correlated with the incidence of pulmonary calcification in tuberculin-negative individuals. The association of positive histoplasmin reactions with calcified nodules in the lungs suggests that, as in the case of coccidioidomycosis, histoplasmosis is not a rare, highly fatal disease, but one which is widespread in certain geographical areas in a mild pulmonary form. That such reactors are or have been actually infected is difficult to demonstrate on any large scale, but infection has been shown in a sufficient number of cases[114] to strongly support such an inference.

Pathogenicity for animals. A variety of animals have been found to be naturally infected, including dogs and cats, wild rodents, and other wild animals.[41] An association with bats and bat caves[40] has been well established. The microorganism has been isolated from bat tissue,[155] but the role of these animals as a reservoir and in dissemination of the infection remains to be determined. It is improbable that these represent an animal reservoir of human infection[112]; rather the fungus appears to be a saprophytic form, living in the soil from which it has been frequently isolated, which infects both man and animals. Mice have, in fact, been infected experimentally by exposure to soil contaminated with the microorganisms.[82] Experimental animals are infected with some facility, a localized lesion being produced at the site of subcutaneous inoculation in guinea pigs and rabbits and generalized infection in dogs and rats. Both yeast-like and mycelial forms are infectious experimentally.

Epidemiology.[58, 59] The infection is air borne and contracted by inhalation. Infection is associated with point sources such as chicken coops, pigeon roosts, bat-infested caves, and old decaying wooden structures where excreta and other decomposing organic matter under conditions of high humidity provide a nidus of infection. The infection tends to occur in rural areas and in low-lying river country. There is a large endemic area of infection in this country in the central-eastern part, including Missouri, Tennessee, Kentucky, Arkansas, and adjacent areas in adjoining states in which the proportion of positive reactors to the histoplasmin test ranges from 50 to 80 per cent. This is in sharp contrast with the areas from the Great Lakes to the Pacific coast, and from Colorado to the Canadian border in which the proportion of reactors is less than 2 per cent. Within the endemic area the proportion of reactors increases with age, from 5 per cent at 2 years, through 60 per cent at 18 years, to 75 to 90 per cent at age 55 and over.

Histoplasmosis is found in Africa where it is known as cave disease because it has often been contracted in caves infested with bats, and differs slightly from American histoplasmosis.[120] Only sporadic cases have been reported from Europe. Consistent with this, the reactor rates are less than 2 per cent except in occasional isolated areas,

such as in Burma and northern Italy, where rates as high as 20 per cent have been observed.

Diagnosis. Microscopic demonstration of the fungus is highly suggestive, but culture and identification are required to establish the diagnosis. *H. capsulatum* has been isolated by blood culture in generalized infections and from biopsy specimens; sternal puncture may prove useful. It may also be isolated from sputum in cases of pulmonary infection by intraperitoneal inoculation of mice.[115] In any case, the mycelial form of the fungus developing on Sabouraud's agar at room temperature is distinguished from other pathogenic fungi by the formation of the large, thick-walled, tuberculate chlamydospores described above.

REFERENCES

1. Abrahams, I., and Gilleran, T. G.: Studies on actively acquired resistance to experimental cryptococcosis in mice. J. Immunol., 1960, **85:**629–635.
2. Ajello, L.: Soil as a natural reservoir for human pathogenic fungi. Science, 1956, **123:** 876–879.
3. Ajello, L.: Occurrence of *Cryptococcus neoformans* in soils. Amer. J. Hyg., 1958, **67:** 72–77.
4. Alznauer, R. L., Rolle, C., Jr., and Pierce, W. F.: Analysis of focalized pulmonary granulomas due to *Coccidioides immitis.* Arch. Path., 1955, **59:**641–655.
5. Baker, R. D.: Mucormycosis—a new disease? J. Amer. Med. Assn., 1957, **163:**805–808.
6. Ball, S., Bessell, C. J., and Mortimer, A.: The production of polyenic antibiotics by soil streptomyces. J. Gen. Microbiol., 1957, **17:** 96–103.
7. Barlow, A. J. E., and Chattaway, F. W.: The parasitism of the ringworm group of fungi. Arch. Dermat., 1958, **77:**399–405.
8. Barrash, M. J., and Fort, M.: Amphotericin B therapy in torula meningitis. Arch. Intern. Med., 1960, **106:**271–274.
9. Baum, G. L.: The significance of *Candida albicans* in human sputum. New England J. Med., 1960, **263:**70–73.
10. Baum, G. L., and Schwarz, J.: North American blastomycosis. Amer. J. Med. Sci., 1959, **238:**661–683.
11. Benham, R. W.: The genus Cryptococcus. Bact. Rev., 1956, **20:**189–201.
12. Benham, R. W., and Kesten, B.: Sporotrichosis: Its transmission to plants and animals. J. Infect. Dis., 1932, **50:**437–458.
13. Binder, L., Csillag, A., and Tóth, G.: Diffuse infiltration of the lungs associated with *Cryptococcus luteolus.* Lancet, 1956, **i:** 1043–1045.
14. Brygoo, E. R., and Segretain, G.: Étude clinique épidémiologique et mycologique de la chromoblastomycose à Madagascar. Bull. Soc. Path. Exot., 1960, **53:**443–475.
15. Burde, R. J. W., and Ainsworth, G. C.: The chemotherapy of fungal diseases. Society for General Microbiology, Symposium No. 8, pp. 309–335. Cambridge University Press, 1958.
16. Burke, R. C., Salvin, S. B., and Gerloff, R. K.: Cultivation of *Coccidioides immitis* in the developing hen's egg. Proc. Soc. Exptl. Biol. Med., 1952, **81:**91–94.
17. Carrión, A. L.: Chromoblastomycosis. Mycologia, 1942, **34:**424–441.
18. Carton, C. A.: Treatment of central nervous system cryptococcosis. A review and report of four cases treated with actidione. Ann. Intern. Med., 1952, **37:**123–154.
19. Cherniss, E. I., and Waisbren, B. A.: North American blastomycosis: A clinical study of 40 cases. Ann. Intern. Med., 1956, **44:**105–123.
20. Christie, A.: The disease spectrum of human histoplasmosis. Ann. Intern. Med., 1958, **49:**544–555.
21. Coe, G. C.: Primary bronchopulmonary aspergillosis. An occupational disease. Ann. Intern. Med., 1945, **23:**324–325.
22. Cohen, A. C.: Pulmonary moniliasis. Amer. J. Med. Sci., 1953, **226:**16–23.
23. Colwell, J. A.: Remission in disseminated coccidioidomycosis produced by amphotericin B. Ann. Intern. Med., 1959, **50:**1028–1035.
24. Conant, N. F.: Cultural study of the life-cycle of *Histoplasma capsulatum,* Darling, 1906. J. Bact., 1941, **41:**563–579.
25. Conant, N. F., and Martin, D. S.: Manual of Clinical Mycology. Saunders, Philadelphia. 2nd ed., 1954.
26. Converse, J. L., and Besemer, A. R.: Nutrition of the parasitic phase of *Coccidioides immitis* in a chemically defined liquid medium. J. Bact., 1959, **78:**231–239.
27. Cope, V. Z.: Visceral actinomycosis. Brit. Med. J., 1949, **ii:**1311–1316.
28. Creitz, J. R., and Puckett, T. F.: A method for cultural identification of *Coccidioides immitis.* Amer. J. Clin. Path., 1954, **24:**1318–1323.
29. Cruickshank, C. N. D., Trotter, M. D., and Wood, S. R.: Studies on trichophytin sensitivity. J. Invest. Dermat., 1960, **35:**219–223.
30. Dalldorf, G. (ed.): Fungi and Fungous Diseases. Thomas, Springfield, Illinois. 1961.
31. Davidson, A. M., and Gregory, P. H.: Note on investigation into fluorescence of hairs infected by certain fungi. Canadian J. Res., 1932, **7:**378–385.
32. Davis, B. L., Smith, R. T., and Smith, C. E.: An epidemic of coccidioidal infection (coccidioidomycosis). J. Amer. Med. Assn., 1942. **118:**1182–1186.
33. Denton, J. J., McConough, E. S., Ajello, L., and Ausherman, R. J.: Isolation of *Blastomyces dermatitidis* from soil. Science, 1961, **133:**1126–1127.
34. Drouhet, E., Hirth, L., and Lebeurier, G.: Some aspects of the mode of action of polyene antifungal antibiotics. Ann. N. Y. Acad. Sci., 1960, **89:**134–155.

35. Drouhet, E., and Schwarz, J.: Comparative studies with 19 strains of Histoplasma. Morphology in tissues and virulence of African and American strains. J. Lab. Clin. Med., 1956, **47:**128–139.
36. Edwards, P. Q., and Klaer, J. H.: World-wide distribution of histoplasmosis and histoplasmin sensitivity. Amer. J. Trop. Med. Hyg., 1956, **6:**235–257.
37. Egeberg, R. O., and Ely, A. F.: *Coccidioides immitis* in the soil of the southern San Joaquin Valley. Amer. J. Med. Sci., 1956, **231:** 151–154.
38. Emmons, C. W.: Medical mycology. Bact. Rev., 1940, **6:**474–514.
39. Emmons, C. W.: Coccidioidomycosis. Mycologia, 1942, **34:**452–463.
40. Emmons, C. W.: Association of bats with histoplasmosis. Pub. Health Repts., 1958, **73:**590–595.
41. Emmons, C. W., et al.: Histoplasmosis. Proved occurrence of inapparent infection in dogs, cats and other animals. Amer. J. Hyg., 1955, **61:**40–44.
42. Emmons, C. W., and Piggott, W.: Amphotericin B and griseofulvin in the treatment of experimental systemic mycoses. Antibiot. Chemotherapy, 1959, **9:**550–556.
43. English, M. P., and Gibson, M. D.: Studies on the epidemiology of tinea pedis. III. Cross-infection in the family. Brit. Med. J., 1960, **i:**1860–1862.
44. Erikson, D.: Pathogenic anaerobic organisms of the actinomyces group. Med. Res. Council (Great Britain) Spec. Rept. Ser. No. 240. 1940.
45. Erikson, D.: The morphology, cytology and taxonomy of the actinomycetes. Ann. Rev. Microbiol., 1949, **3:**23–54.
46. Evans, E. E.: The antigenic composition of *Cryptococcus neoformans.* I. A serologic classification by means of the capsular and agglutination reactions. J. Immunol., 1950, **64:**423–430.
47. Evans, E. E., and Kessel, J. F.: The antigenic composition of *Cryptococcus neoformans.* II. Serologic studies with the capsular polysaccharide. J. Immunol., 1951, **67:**109–114.
48. Evans, E. E., and Theriault, R. J.: The antigenic composition of *Cryptococcus neoformans.* IV. The use of paper chromatography for following purification of the capsular polysaccharide. J. Bact., 1953, **65:**571–577.
49. Fetter, B. F.: Human cutaneous sporotrichosis due to *Sporotrichum schenckii:* Technique for demonstration of organisms in tissues. Arch. Path., 1961, **71:**416–419.
50. Fiese, M. J.: Coccidioidomycosis. Thomas, Springfield, Illinois. 1958.
51. Frey, D. M., and Durie, E. B.: The isolation of keratinophilic fungi, including *Microsporum gypseum,* from Australian soil. Australian J. Exptl. Biol. Med. Sci., 1956, **34:**199–204.
52. Friedman, L., and Conant, N. F.: Immunologic studies on the etiologic agents of North and South American blastomycosis. I. Comparison of hypersensitivity reactions. Mycopathologia, 1953, **6:**310–316.
53. Friedman, L., and Conant, N. F.: Immunologic studies on the etiologic agents of North and South American blastomycosis. II. Comparison of serologic reactions. Mycopathologia, 1953, **6:**317–324.
54. Friedman, L., Derbes, V. J., Hodges, E. P., and Sinski, J. T.: The isolation of dermatophytes from the air. J. Invest. Dermat., 1960, **35:**3–5.
55. Friedman, L., Smith, C. E., and Gordon, L. E.: The assay of virulence of Coccidioides in white mice. J. Infect. Dis., 1955, **97:**311–316.
56. Fuentes, C. A., Bosch, Z. E., and Boudet, C. C.: The isolation of *Microsporum gypseum* from soil. Arch. Dermat., 1955, **71:** 684–687.
57. Fuentes, C. A., Schwarz, J., and Aboulafia, R.: Some aspects of the pathogenicity of *Candida albicans* in laboratory animals. Mycopathologia, 1952, **6:**176–181.
58. Furcolow, M. L.: Airborne histoplasmosis. Bact. Rev., 1961, **25:**301–309.
59. Furcolow, M. L., and Ney, P. E.: Epidemiologic aspects of histoplasmosis. Amer. J. Hyg., 1957, **65:**264–270.
60. Gale, D., and Waldron, C. A.: Experimental actinomycosis with *Actinomyces israeli.* The development of visceral actinomycosis. J. Infect. Dis., 1955, **97:**251–261.
61. Gastineau, F. M., Spolyar, L. W., and Haynes, E.: Sporotrichosis. Report of six cases among florists. J. Amer. Med. Assn., 1941, **117:**1074–1077.
62. Georg, L. K., and Camp, L. B.: Routine nutritional tests for the identification of dermatophytes. J. Bact., 1957, **74:**113–121.
63. Gold, W., Stout, H. A., Pagano, J. S., and Donovick, R.: Amphotericins A and B: Antifungan antibiotics produced by a streptomycete. I. In vitro studies. Antibiotics Annual, 1955–56, pp. 579–586. Medical Encyclopedia, New York. 1956.
64. Goldstein, D. M., and Louie, S.: Primary pulmonary coccidioidomycosis. Report of an epidemic of seventy-five cases. War Med., 1943, **4:**299–317.
65. Gonzáles Bonavides, J.: Sporotrichosis as an occupational disease in workpeople at potteries. Bol. Soc. Cubana de Dermat. y Sifil., 1956, **13:**89–102.
66. Gordon, D. M.: Ocular sporotrichosis: Report of a case. Arch. Ophthal., 1947, **37:**56–72.
67. Gordon, M. A.: A key to the human mycoses. J. Bact., 1952, **63:**385–392.
68. Grcevic, N., and Matthews, W. F.: Pathologic changes in acute disseminated aspergillosis. Particularly involvement of the central nervous system. Amer. J. Clin. Path., 1959, **32:** 536–551.
69. Gregory, P. H.: The dermatophytes. Biol. Rev., 1935, **10:**208–233.
70. Harrell, E. R., Bocobo, F. C., and Curtis, A. C.: A study of North American blastomycosis and its treatment with stilbamidine and 2-hydroxystilbamidine. Ann. Intern. Med., 1955, **43:**1076–1091.
71. Harrell, E. R., and Curtis, A. C.: The treatment of North American blastomycosis with amphotericin B. Arch. Dermat., 1957, **76:** 561–568.
72. Harvey, J. C., Cantrell, J. R., and Fisher, A. M.: Actinomycosis: Its recognition and treatment. Ann. Intern. Med., 1957, **46:** 868–885.
73. Hasenclever, H. F., and Mitchell, W. O.: Antigenic studies of Candida. I. Observation of

two antigenic groups in *Candida albicans*. J. Bact., 1961, **82:**570–573.
74. Hasenclever, H. F., and Mitchell, W. O.: Antigenic studies of Candida. III. Comparative pathogenicity of *Candida albicans* group A, group B and *Candida stellatoidea*. J. Bact., 1961, **82:**578–581.
75. Hasenclever, H. F., Mitchell, W. O., and Loewe, J.: Antigenic studies of Candida. II. Antigenic relation of *Candida albicans* group A and group B to *Candida stellatoidea* and *Candida tropicalis*. J. Bact., 1961, **82:**574–577.
76. Haugen, R. K., and Baker, R. D.: The pulmonary lesions in cryptococcosis with special reference to subpleural nodules. Amer. J. Clin. Path., 1954, **24:**1381–1390.
77. Hazen, E. L., and Brown, R.: Nystatin. Ann. N. Y. Acad. Sci., 1960, **89:**258–266.
78. Hazen, E. L., and Reed, F. C.: Laboratory Identification of Pathogenic Fungi Simplified. Thomas, Springfield, Illinois. 1960.
79. Henrici, A. T.: An endotoxin from *Aspergillus fumigatus*. J. Immunol., 1939, **36:**319–338.
80. Henrici, A. T.: The yeasts. Genetics, cytology, variation, classification and identification. Bact. Rev., 1941, **5:**97–179.
81. Hensler, N. M., and Cleve, E. A.: Chronic benign residuals of coccidioidomycosis. Arch. Intern. Med., 1956, **98:**61–70.
82. Hinton, A., Larsh, H. W., and Silberg, S. L.: Direct exposure of mice to soils known to contain *Histoplasma capsulatum*. Proc. Soc. Exptl. Biol. Med., 1957, **94:**176–179.
83. Hosty, T. S., et al.: Prevalence of *Nocardia asteroides* in sputa examined by a tuberculosis diagnostic laboratory. J. Lab. Clin. Med., 1961, **58:**107–114.
84. Hunter, R. C., Jr., and Mongan, E. S.: Disseminated coccidioidomycosis treated with amphotericin B. U. S. Armed Forces Med. J., 1958, **9:**1474–1486.
85. Huntington, R. W., Jr.: Morphology and racial distribution of fatal coccidioidomycosis. Report of a ten-year autopsy series in an endemic area. J. Amer. Med. Assn., 1959, **169:**115–118.
86. Huppert, M., and Cazin, J., Jr.: Pathogenesis of *Candida albicans* infection following antibiotic therapy. II. Further studies of the effect of antibiotics on the in vitro growth of *Candida albicans*. J. Bact., 1955, **70:**435–439.
87. Huppert, M., Cazin, J., Jr., and Smith, H., Jr.: Pathogenesis of *Candida albicans* infection following antibiotic therapy. III. The effect of antibiotics on the incidence of *Candida albicans* in the intestinal tract of mice. J. Bact., 1955, **70:**440–447.
88. Huppert, M., MacPherson, D. S., and Cazin, J., Jr.: Pathogenesis of *Candida albicans* infection following antibiotic therapy. I. The effect of antibiotics on the growth of *Candida albicans*. J. Bact., 1953, **65:**171–176.
89. Kao, C. J., and Schwarz, J.: The isolation of *Cryptococcus neoformans* from pigeon nests. With remarks on the identification of virulent cryptococci. Amer. J. Clin. Path., 1957, **27:**652–663.
90. Karrer, H. E.: Virulence of *Coccidioides immitis* determined by intracerebral inoculation in mice. Proc. Soc. Exptl. Biol. Med., 1953, **82:**766–768.
91. Kase, A., and Marshall, J. D., Jr.: A study of *Cryptococcus neoformans* by means of the fluorescent antibody technic. Amer. J. Clin. Path., 1960, **34:**52–56.
92. Kligman, A. M.: Tinea capitis due to *M. aoudouini* and *M. canis*. II. Dynamics of the host-parasite relationship. Arch. Dermat., 1955, **71:**313–337.
93. Kligman, A. M., and DeLamater, E. D.: The immunology of the human mycoses. Ann. Rev. Microbiol., 1950, **4:**283–312.
94. Kuykendall, S. J., Ellis, F. H., Jr., Weed, L. A., and Donoghue, F. E.: Pulmonary cryptococcosis. New England J. Med., 1957, **257:**1009–1016.
95. Lacaz, C. da S.: Manual de micologia medica. Livraria Atheneu S/A, Rio de Janeiro. 3rd ed., 1960.
96. Larsh, H. W., Hinton, A., and Silberg, S. L.: Conversion and maintenance of *Histoplasma capsulatum* in tissue culture. Proc. Soc. Exptl. Biol. Med., 1956, **93:**612–615.
97. La Touche, C. J.: The importance of the animal reservoir of infection in the epidemiology of animal-type ringworm in man. Vet. Record, 1955, **67:**666.
98. Lewis, G. M., Hopper, M. E., Wilson, J. W., and Plunkett, O.: Introduction to Medical Mycology. Year Book Publishers, Chicago. 4th ed., 1958.
99. London, I. D.: Hydroxystilbamidine treatment of North American blastomycosis. Southern Med. J., 1956, **49:**1098–1103.
100. Lones, G. W., and Peacock, C. L.: Role of carbon dioxide in the dimorphism of *Coccidioides immitis*. J. Bact., 1960, **79:**308–309.
101. Mackinnon, J. E.: Pathogenesis of South American blastomycosis. Trans. Roy. Soc. Trop. Med. Hyg., 1959, **53:**487–494.
102. Mackinnon, J. E., and Artagaveytia-Allende, R. C.: Aspectos actuales de la moniliasis. An. Facul. Med. Montevideo, 1956, **41:**275–292.
103. Maddy, K. T.: Coccidioidomycosis of cattle in the southwestern United States. J. Amer. Vet. Med. Assn., 1954, **124:**456–464.
104. Many, H., Derbes, V. J., and Friedman, L.: *Trichophyton rubrum:* Exposure and infection within household groups. Arch. Dermat., 1960, **82:**226–229.
105. Martin, D. S.: Practical applications of immunologic principles in the diagnosis and treatment of fungus infections. Ann. N. Y. Acad. Sci., 1950, **50:**1376–1379.
106. Martin, D. S.: Serologic studies on North American blastomycosis. Studies with soluble antigens from untreated and sonic-treated yeast-phase cells of *Blastomyces dermatitidis*. J. Immunol., 1953, **71:**192–201.
107. Martin, D. S., and Smith, D. T.: The laboratory diagnosis of blastomycosis. J. Lab. Clin. Med., 1937, **21:**1289–1296.
108. Martin, D. S., and Smith, D. T.: Blastomycosis (American blastomycosis, Gilchrist's disease). I. A review of the literature. Amer. Rev. Tuberc., 1939, **39:**488–515.
109. Martin, W. J., and Nichols, D. R.: The mycoses. Proc. Staff Meetings Mayo Clinic, 1960, **35:**149–161.
110. McDonough, E. S. et al.: Human pathogenic fungi recovered from soil in an area endemic for North American blastomycosis. Amer. J. Hyg., 1961, **73:**75–83.
111. Meame, P., and Rayner, D.: Mucormycosis. A report on twenty-two cases. Arch. Path., 1960, **70:**261–268.

112. Menges, R. W., Furcolow, M. L., and Hinton, A.: The role of animals in the epidemiology of histoplasmosis. Amer. J. Hyg., 1954, **59:** 113–118.
113. Menges, R. W., and Georg, L. K.: Survey of animal ringworm in the United States. Pub. Health Repts., 1957, **72:**503–509.
114. Mochi, A., and Edwards, P. Q.: Geographical distribution of histoplasmosis and histoplasmin sensitivity. Bull. W. H. O., 1952, **5:**259–291.
115. Moffit, G. W., Morgan, B. H., and Cameron, G. M.: The isolation of *Histoplasma capsulatum* from sputum as a diagnostic procedure. J. Lab. Clin. Med., 1956, **47:**499–502.
116. Mortimer, P. H.: Man, animals and ringworm. Vet. Record, 1955, **67:**670.
117. Mosberg, W. H., Jr., and Arnold, J. G., Jr.: Torulosis of the central nervous system: Review of the literature and report of five cases. Ann. Intern. Med., 1950, **32:**1153–1183.
118. Moss, E. S., and McQuown, A. L.: Atlas of Medical Mycology. Williams and Wilkins, Baltimore. 2nd ed., 1960.
119. Murray, I. G., Spooner, E. T. C., and Walker, J.: Experimental infection of mice with *Madurella mycetomi.* Trans. Roy. Soc. Trop. Med. Hyg., 1960, **54:**335–341.
120. Murray, J. F., Lurie, H. I., and Brandt, F. A.: Histoplasmosis in Africa. J. Trop. Med. Hyg., 1958, **61:**124–130.
121. Naji, A. F.: Bronchopulmonary aspergillosis. Report of two new cases, review of the literature, and suggestion for classification. Arch. Path., 1959, **68:**282–291.
122. Negroni, P.: Micosis profundas (cutáneas y viscerales). vol. II. Histoplasmosis. Histoplasmosis en La Argentina. Comisión de Investigación Científica, La Plata. 1960.
123. Newcomer, V. D. et al.: The treatment of systemic fungus infections with amphotericin B. Ann. N. Y. Acad. Sci., 1960, **89:**221–246.
124. Nichols, D. R., and Martin, W. J.: Cryptococcosis: Clinical features and differential diagnosis. Ann. Intern. Med., 1955, **43:**767–780.
125. Nickerson, W. J. (ed.): Biology of Pathogenic Fungi. Chronica Botanica, Waltham, Massachusetts. 1947.
126. Nickerson, W. J.: Medical mycology. Ann. Rev. Microbiol., 1953, **7:**245–272.
127. Norden, Å.: Sporotrichosis. Clinical and laboratory features and a serological study in experimental animals and humans. Acta Path. Microbiol. Scand., 1951, Suppl. 89.
128. O'Leary, D. J., and Curry, F. J.: Coccidioidomycosis. A review and presentation of 100 consecutively hospitalized patients. Amer. Rev. Tuberc., 1956, **73:**501–518.
129. Oroshnik, W., Vining, L. C., Mebane, A. D., and Taber, W. A.: Polyene antibiotics. Science, 1955, **121:**147–149.
130. Puckett, T. F.: Hyphae of *Coccidioides immitis* in tissues of the human host. Amer. Rev. Tuberc., 1954, **70:**320–327.
131. Rapaport, F. T., et al.: The immunologic properties of coccidioidin as a skin test reagent in man. J. Immunol., 1960, **84:**368–373.
132. Reiss, F., Kornblee, L., and Gordon, B.: Griseofulvin (Fulvicin) in treatment of fungous infections of the hair, skin and nails with special reference to mycologic changes produced during therapy. J. Invest. Dermat., 1960, **34:**263–269.
133. Ridley, M. F.: A comparison of methods for identification of *Candida albicans.* Australian J. Dermat., 1960, **5:**209–213.
134. Ridley, M. F.: The isolation of keratinophilic fungi from animals and soil in Queensland. Australian J. Dermat., 1961, **6:**29–32.
135. Rieth, H., Schönfeld, J., and Schirren, C.: Beziehungen zwischen humanen und animalen Dermatomykosen. 4. Mitteilung. Sollen spontan heilende Mikrosporien bei Katzen und Hunden wie beim Menschen behandelt werden. Bull. Pharmaceut. Res. Inst., 1960, No. 28, pp. 1–8.
136. Robinson, H. M., Jr., et al.: Griseofulvin, clinical and experimental studies. Arch. Dermat., 1960, **81:**66–76.
137. Robinson, H. M., Jr., Figge, F. H. J., and Bereston, E. S.: Fluorescence of *Microsporum audouini*-infected hair. I. Chemical and spectroscopic studies. Arch. Dermat. Syph., 1953, **68:**129–135.
138. Robinson, R. C. V.: Cutaneous moniliasis in infants. J. Pediatrics, 1957, **50:**721–723.
139. Roessler, W. G., Converse, J. L., and Geating, J. A.: Virulence assay of *Coccidioides immitis* in embryonated eggs. J. Bact., 1961, **81:** 226–232.
140. Rosebury, T.: The parasitic actinomycetes and other filamentous microorganisms of the mouth. Bact. Rev., 1944, **8:**190–223.
141. Rosebury, T., Epps, L. J., and Clark, A. R.: A study of the isolation, cultivation and pathogenicity of *Actinomyces israeli* recovered from the human mouth and from actinomycosis in man. J. Infect. Dis., 1944, **74:**131–149.
142. Roth, F. J., Jr.: Griseofulvin. Ann. N. Y. Acad. Sci., 1960, **89:**247–253.
143. Salvin, S. B., and Smith, R. F.: An antigen for detection of hypersensitivity to *Cryptococcus neoformans.* Proc. Soc. Exptl. Biol. Med., 1961, **108:**498–501.
144. Schneidau, J. D., Jr., and Shaffer, M. F.: Studies on Nocardia and other Actinomycetales. I. Cultural studies. Amer. Rev. Tuberc., 1957, **76:**770–788.
145. Schulz, D. M.: Histoplasmosis of the central nervous system. J. Amer. Med. Assn., 1953, **151:**549–551.
146. Schulz, D. M.: Histoplasmosis: A statistical morphologic study. Amer. J. Clin. Path., 1954, **24:**11–26.
147. Schwartz, B.: Histoplasmosis of lungs. Arch. Intern. Med., 1954, **94:**970–994.
148. Schwartzman, R. M., Fusaro, R. M., and Orkin, M.: Transmission of North American blastomycosis: Possible case of transmission from dog to human. J. Amer. Med. Assn., 1959, **171:**2185–2189.
149. Schwarz, J., and Baum, G. L.: Blastomycosis. Amer. J. Clin. Path., 1951, **21:**999–1029.
150. Schwarz, J., and Baum, G. L.: North American blastomycosis. (Geographic distribution, pathology and pathogenesis.) Documenta Med. Geograph. Trop., 1953, **5:**29–41.
151. Schwarz, J., and Goldman, L.: Epidemiologic study of North American blastomycosis. Arch. Dermat., 1955, **71:**84–88.
152. Schwarz, J., and Loutzenhiser, J. K.: Laboratory experiences with griseofulvin. J. Invest. Dermat., 1960, **34:**295–299.
153. Scott, S. M., Peasley, E. D., and Crymes, T. P.: Pulmonary sporotrichosis. Report of two cases with cavitation. New England J. Med., 1961, **265:**453–457.

154. Seabury, J. H., and Dascomb, H. E.: Experience with amphotericin B. Ann. N. Y. Acad. Sci., 1960, **89:**202–220.
155. Shacklette, M. H., Diercks, F. H., and Gale, N. B.: *Histoplasma capsulatum* recovered from bat tissues. Science, 1962, **135:**1135.
156. Shapiro, J. L., Lux, J. J., and Sprofkin, B. E.: Histoplasmosis of the central nervous system. Amer. J. Path., 1955, **31:**319–335.
157. Skinner, C. E., Emmons, C. W., and Tsuchiya, H. H.: Henrici's Molds, Yeasts and Actinomycetes. John Wiley and Sons, New York. 2nd ed., 1947.
158. Skinner, C. E., and Fletcher, D. W.: A review of the genus Candida. Bact. Rev., 1960, **24:** 397–416.
159. Slack, J. M.: The source of infection in actinomycosis. J. Bact., 1942, **43:**193–209.
160. Slack, J. M., Ludwig, E. H., Bird, H. H., and Canby, C. M.: Studies with microaerophilic actinomycetes. I. The agglutination reaction. J. Bact., 1951, **61:**721–735.
161. Slack, J. M., Spears, R. G., Snodgrass, W. G., and Kuchler, R. J.: Studies with microaerophilic actinomycetes. II. Serological groups as determined by the reciprocal agglutinin absorption technique. J. Bact., 1955, **70:**400–404.
162. Slack, J. M., Winger, A., and Moore, D. W., Jr.: Serological grouping of Actinomyces by means of fluorescent antibodies. J. Bact., 1961, **82:**54–65.
163. Small, M. J.: Late progression of pulmonary coccidioidomycosis. Arch. Intern. Med., 1959, **104:**730–740.
164. Smith, C. E., et al.: Comparison of complement fixation tests for coccidioidomycosis. Pub. Health Repts., 1957, **72:**888–894.
165. Smith, C. E., Beard, R. R., Whiting, E. G., and Rosenberger, H. G.: Varieties of coccidioidal infection in relation to the epidemiology and control of the diseases. Amer. J. Pub. Health, 1946, **36:**1394–1402.
166. Smith, C. E., Pappagianis, D., Levine, H. B., and Saito, M.: Human coccidioidomycosis. Bact. Rev., 1961, **25:**310–320.
167. Smith, D. E., and Matthews, J. H.: Treatment of pulmonary mycotic infections with amphotericin B. New England J. Med., 1960, **263:** 782–784.
168. Smith, G. W., et al.: Cryptococcosis of the central nervous system: Four cases treated with amphotericin B. Southern Med. J., 1960, **53:**305–311.
169. Smith, J. G., Jr., Harris, J. S., Conant, N. F., and Smith, D. T.: An epidemic of North American blastomycosis. J. Amer. Med. Assn., 1955, **158:**641–646.
170. Stockdale, P. M.: Nutritional requirements of the dermatophytes. Biol. Rev., 1953, **28:**84–104.
171. Stockdale, P. M.: *Nannizzia incurvata* gen. nov., sp. nov., a perfect state of *Microsporum gypseum* (Bodin) Guiart et Grigorakis. Sabouraudia, 1961, **1:**41–48.
172. Straub, M., and Schwarz, J.: The healed primary complex in histoplasmosis. Amer. J. Clin. Path., 1955, **25:**727–741.
173. Straub, M., and Schwarz, J.: Primary pulmonary arrested lesions of coccidioidomycosis and histoplasmosis. A study of autopsy material in Tucson, Arizona. Amer. J. Clin. Path., 1956, **26:**998–1009.
174. Sutliff, W. D.: Fungal infections and chemotherapy. Ann. Rev. Med., 1958, **9:**15–30:
175. Sutliff, W. D., Kyle, J. W., and Hobson, J. L.: North American blastomycosis: Clinical forms of the disease and treatment with stilbamidine and 2-hydroxystilbamidine. Ann. Intern. Med., 1954, **41:**89–107.
176. Swartz, J. H.: Elements of Medical Mycology. Saunders, Philadelphia. 2nd ed., 1954.
177. Symposium: Medical mycology. Ann. N. Y. Acad. Sci., 1950, **50:**1209–1404.
178. Symposium: Proceedings of the Conference on Histoplasmosis held at Excelsior Springs, Missouri, November 18–20, 1952. Public Health Monograph No. 39. Public Health Service, U. S. Department of Health, Education and Welfare. 1956.
179. Symposium: Cryptococcosis: Clinical staff conference at the National Institutes of Health. Ann. Intern. Med., 1958, **49:**642–661.
180. Symposium: Second conference on medical mycology. Ann. N. Y. Acad. Sci., 1960, **89:** 1–282.
181. Symposium: Griseofulvin and dermatomycoses: An international symposium sponsored by the University of Miami, October 26–27, 1959. Arch. Dermat., 1960, **81:**649–882.
182. Takos, M. J.: Experimental cryptococcosis produced by the ingestion of virulent organisms. New England J. Med., 1956, **254:**598–601.
183. Tate, P.: Dermatophytes or ringworm fungi. Biol. Rev., 1929, **4:**41–75.
184. Tiant, F. R., and Fuentes, C. A.: Cryptococcosis de localización cutánea. Bol. Soc. Cubana de Dermat y Sifil., 1958, **15:**65–72.
185. Tsuchiya, T., Iwahara, S., Mikasaki, F., and Fukazawa, Y.: Studies on the classification of genus Candida. Antigenic analysis of seven species of genus Candida. Japanese J. Exptl. Med., 1954, **24:**95–103.
186. Ueckert, H.: Beitrag zur Systematik der Dermatophyten. Zent. f. Bakt., I Abt. Orig., 1959, **176:**102–156.
187. Vignec, A. J.: The role of *Candida albicans* in the common skin disorders of infancy and early childhood. J. Pediatrics, 1958, **53:**692–703.
188. Vogel, R. A., Peace, R. J., and Koger, M. T.: Histopathologic reactions of the yolk sac tissue of embryonated hen's eggs to *Coccidiodes immitis*. Amer. J. Path., 1957, **33:** 1023–1033.
189. Waksman, S. A.: Species concept among the actinomycetes with special reference to the genus Streptomyces. Bact. Rev., 1957, **21:** 1–29.
190. Waksman, S. A., and Henrici, A. T.: The nomenclature and classification of the actinomycetes. J. Bact., 1943, **46:**337–341.
191. Webster, B. H.: Pulmonary nocardiosis. A review with a report of seven cases. Amer. Rev. Tuberc., 1956, **73:**485–500.
192. Weed, L. A., Anderson, H. A., Good, C. A., and Baggenstoss, A. H.: Nocardiosis. Clinical, bacteriologic and pathological aspects. New England J. Med., 1955, **253:**1137–1143.
193. Ziprkowski, L., Krakowski, A., Schewach-Millet, M., and Btesh, S.: Griseofulvin in the mass treatment of tinea capitis. Bull. W. H. O., 1960, **23:**803–810.

Chapter 33

THE SPIROCHETES

Spirally shaped microorganisms were for a long time grouped together indifferently under the common name of spirillum, spirochete, or vibrio. Further knowledge has shown that some of these are true bacteria, although curved, and such forms are now placed with the bacteria in the genus Vibrio and various species of Spirillum. Others, however, possess characteristics which distinguish them from curved bacteria, such as the cholera vibrio, and also from the actively motile protozoa, such as the trypanosomes. Like the bacteria, the spirochetes multiply by transverse fission, have no well-defined nucleus, and do not exhibit anterior-posterior polarity. They appear to be more closely related to the true bacteria than to the protozoa and may, perhaps, be regarded as a connecting link between the two.[108]

Classification and nomenclature within the group are still in an uncertain condition, and there is by no means general agreement regarding the separation of some of the genera. These microorganisms are difficult to cultivate on artificial mediums in many cases, and little or nothing is known of their physiological characteristics; the genera are separated from one another on a purely morphological basis.[31] Studies by electron microscopy have tended to confirm the soundness of the morphological criteria.[102]

Although the name spirochete is commonly used to designate those forms which are classified as members of the family Spirochaetaceae, the generic term Spirochaeta was originally proposed by Ehrenberg for the large free-living forms he described. A second genus, Cristispira, is characterized by the presence of a membranous appendage or crista wound about the body of the cell and is made up of microorganisms found living as saprophytic commensals in certain molluscs. A third genus, Saprospira, consists of free-living forms which differ from Cristispira in that the crista is absent. The other genera contain the spirochetes which are pathogenic for man and animals: Borrelia, the spirochetes of the relapsing fevers; Leptospira, the spirochetes causing Weil's disease or infectious jaundice and certain fevers; and Treponema, the spirochetes of syphilis and yaws.

THE SPIROCHETES OF THE RELAPSING FEVERS (BORRELIA)[50, 76, 104]

The relapsing fevers are a group of closely related infections characterized clinically by an initial pyrexia of 3 to 4 days' duration, followed at intervals of a few days by successive relapses. They are widely distributed and occur in

every country of the world with the possible exception of Australia. The microorganisms responsible for these diseases are spiral forms, first observed by Obermeier in 1873 in the blood of patients with European relapsing fever.

Morphology. The basic structure of these spirochetes is a spring-like axial filament upon which there is a layer of contractile protoplasm enveloped in a delicate periplast. The terminal filaments are, perhaps, drawn-out ends of the periplast or nonrigid and noncoiled ends of the axial filament. Attached spherical bodies are sometimes observed. The cell rotates as a consequence of the stretching of the axial filament by the pressure of contracting protoplasm. When there are rapid and successive contractions and relaxations, the microorganism rotates rapidly and moves in one direction or another according to the direction of the rotation. In the relaxed state the spirals are regular and even; the larger the cell the heavier the axial filament and the greater the distance between the spirals. The regularity of the spirals is disturbed by the contracting and relaxing protoplasm in the actively motile cells. The various species cannot be distinguished from one another on a morphological basis. All are 0.2 to 0.5 μ in breadth by 10 to 20 μ in length. They are best stained by the Romanowsky method or some modification such as Giemsa, but, in contrast to some of the others, Borrelia may be stained with the ordinary aniline dyes.

Cultivation. The spirochetes of relapsing fever were cultivated by Noguchi in 1912 in a medium of ascitic fluid and fresh tissue in which a network of fibrin is formed. A semisolid serum agar has been used also. In culture the spirochetes are aerobic, and the paraffin-oil seal frequently used prevents evaporation rather than interferes with the diffusion of oxygen. While such successful cultivation has been reported, the spirochetes are exceeding difficult to grow in initial culture and cannot be maintained in serial passage; it is not unlikely that they only persist for a time in such mediums and never have been actually cultivated. They may be grown in the developing chick embryo, however, and have been isolated directly from human infections in this way.[13, 87]

Classification. The relapsing fever spirochetes have been given the generic name Borrelia and are so called in the Bergey classification. The validity of this degree of differentiation from the spirochetes of syphilis and yaws, Treponema, is open to some question, and the relapsing fever spirochetes are regarded by many as Treponema species. It was pointed out by Noguchi that the relapsing fever spirochetes do not differ more from *Treponema pertenue* (yaws) than

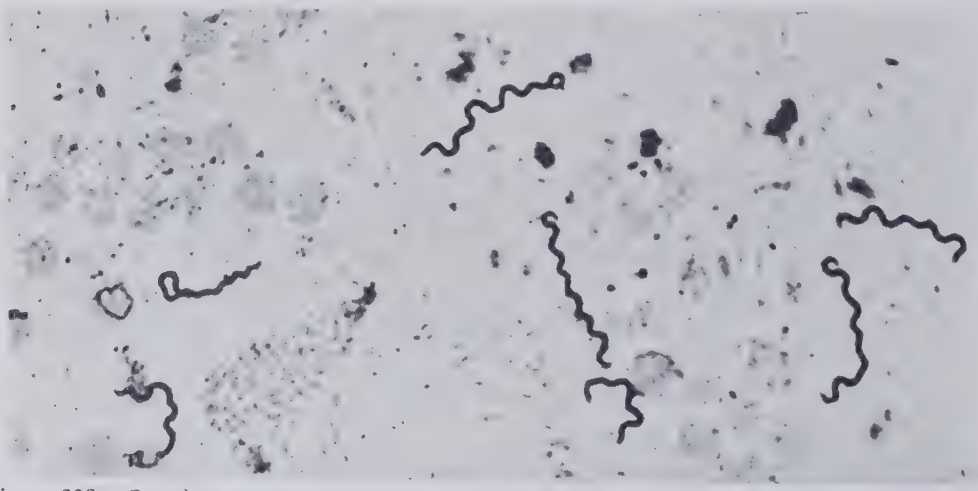


Figure 223. *Borrelia recurrentis* in a blood smear from an infected rat. Fontana stain; × 1160. (Freeman.)

Figure 224. Relapsing fever spirochetes. *Left, Borrelia duttonii* of Central African relapsing fever: *right, Borrelia kochii* of East African relapsing fever. × 2000. (Kral.)

that microorganism differs from *T. pallidum.*

It is not clear to what extent the relapsing fever spirochetes can be divided into species. It is probable that in many cases those given species rank are, in fact, only strains or varieties. In many instances these have been given place names or named after some individual worker, but the differentiation has been geographical rather than biological. These forms are immunologically heterogeneous and unstable in that they are modified in character by residence in different insect or mammalian hosts. Cross-immunity tests, therefore, are not useful. It has been suggested that they are differentiated on the basis of pathogenicity or insect host, but there appear to be no reasonably definite lines of demarcation possible on these bases. In general, then, the questions of interrelationship and speciation of these organisms are almost completely open but most workers agree that, although they are all immunologically related, there is too great heterogeneity to justify regarding the various strains as no more than varieties of a single species *Borrelia* (*Treponema*) *recurrentis.*

Pathogenicity. Relapsing fever is an ancient disease, and one of the first epidemics was described by Hippocrates as occurring on the island of Thasos 2000 years ago. It was not mentioned for many centuries, and then there was a tendency to confuse it with louse-borne typhus fever with which it may occur. The Yellow Plague that followed the pandemic of bubonic plague in the time of Justinian was probably relapsing fever, and an epidemic in Ireland in 664 was described by the Venerable Bede.[64, 69] At the present time it occurs sporadically in various parts of the world.

As indicated above, the relapsing fevers constitute a group of closely related diseases. The spirochete observed by Obermeier was that of European relapsing fever, a disease which has been known since the early part of the eighteenth century and has at times prevailed extensively in parts of Europe. The causal microorganism is designated *Borrelia* (or *Treponema*) *recurrentis.* Four forms of relapsing fever occur in Africa; the spirochete of the form in Central Africa is *B. duttonii* which is also found in the Middle East[1]; that of the East African disease *B. kochii;* that occurring in northern Africa,[32] in Tunis, Algiers, and Tripoli is *B. berbera;* and that in the Sudan *B. aegyptica,* and *Borrelia hispanica* is found in Spain and North Africa. Relapsing fever also occurs in India, and the causative microorganism is *B. carteri.* American relapsing fever is caused by still another spirochete, *B. novyi;* other strains isolated in this country have been named *B. turicatae.* South American strains have been called *B. venezuelensis* and *B. neotropicalis.*

All forms of relapsing fever are clin-

ically identical. The onset is sudden, with chills, fever, and severe headache; muscular and joint pains are frequently observed, and there is a moderate enlargement and tenderness of the spleen and commonly jaundice. The fever ends suddenly by crisis in 3 or 4 days. Successive relapses occur at intervals of 2 to 14 days, and the period of relapse varies from a few hours to longer than the primary fever. The number of relapses varies. Spirochetes may be found in the blood during the paroxysms. The case fatality is not high in European relapsing fever, perhaps 4 to 5 per cent. There are no characteristic findings at autopsy.

The infection may be transmitted to the rat and mouse, but the guinea pig appears resistant to most strains though it may be infected with certain American strains. Neonatal guinea pigs have been found to be more susceptible, and the susceptibility is differential with respect to at least some strains.[9] It has been reported that the hamster may be infected with at least some strains. It is of some importance that the blood for inoculation be taken at the onset rather than at the decline of the relapse. Ordinarily the infection is inapparent in both rats and mice in that there are no observable symptoms, but successive "attacks," beginning 2 to 4 days after inoculation, are evidenced by the appearance of the spirochetes in the blood. They are present for 2 to 3 days, disappear, and reappear 3 or 4 days later in a second "attack." Usually only two or three such relapses occur, the spirochetes becoming fewer and persisting for shorter times in the successive relapses. Experimental disease may also be produced in monkeys, and its course is very similar to that in man. There is some evidence that tetracyclines may have utility as chemotherapeutic agents.

Natural infection of lower animals occurs with some frequency in endemic areas, and there is much evidence to support the hypothesis that small mammals, especially rodents, constitute a natural reservoir of the tick-borne infection. A wide variety of animals may be infected, including the rat, mouse, chipmunk, squirrel, opossum, porcupine, armadillo, hedgehog, fox, and dog. Susceptibility varies considerably among animal species, and from one strain to another of the spirochete.

A spirochetosis of geese is due to *B. anserinum,* a microorganism regarded by many as identical with *B. gallinarum,* which produces septicemia of chickens. Another species, *B. theileri,* is associated with a benign affection of cattle in South Africa but appears to have but low pathogenic powers. Morphologically similar spirochetes have also been found in the blood of sheep, horses, and bats and in the alimentary tract of fish and insects, but they are not associated with any obvious indisposition.

Immunity. Following infection, agglutinins and lytic and spirocheticidal antibodies may be found in the serum. Usually the immunity following recovery from an attack of relapsing fever is of short duration, but occasionally a more solid immunity may develop. The latter is often attributed to a persistence of the infection, *i.e.,* an immunity to superinfection. This is not definitely known, however.

Relapse appears to be basically an immunological phenomenon and dependent upon the antigenic instability of the spirochete. It has been shown that the spirochetes will remain alive for as long as 40 days in blood drawn before the onset of an attack, but in blood drawn during the decline of an attack or after recovery they die out in less than an hour. In the latter case the blood is spirocheticidal and the killed spirochetes are phagocytosed. This has been taken to suggest that the relapse is a consequence of the survival of a few individuals which are resistant to the specific spirocheticidin and which multiply to give rise to a new serum-fast strain. It has been found that spirochetes isolated after successive relapses differ serologically from the spirochetes of the first attack, and as many as nine different serological types of *B. carteri* were distinguished by Cunningham; after any particular type appeared in an animal it

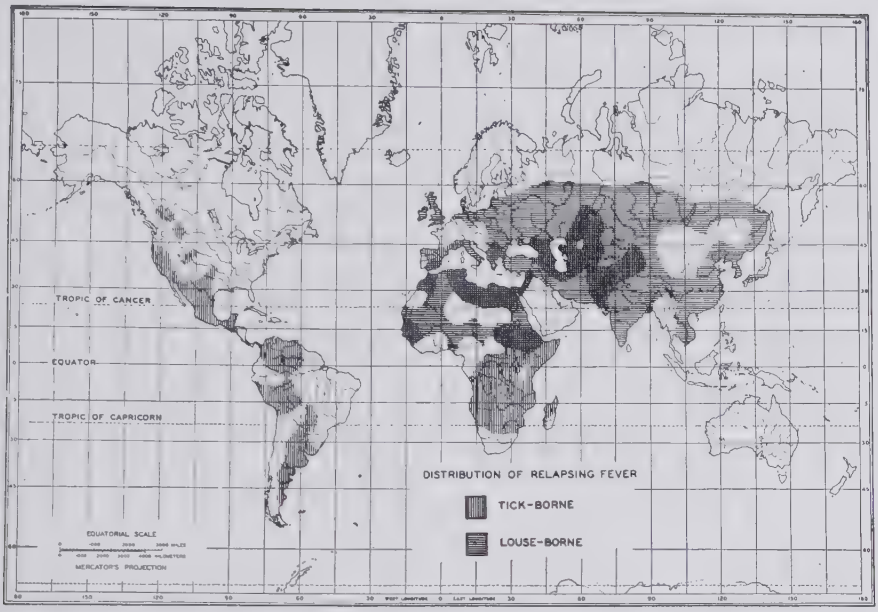


Figure 225. The world-wide distribution of relapsing fever, both louse- and tick-borne. Redrawn from map prepared by Army Medical Intelligence, 1943. Based on Goode *Base Map* No. 201 M. By permission of the University of Chicago Press.

never reappeared in the further course of the disease in the same animal.[95] It is not clear whether this immunological variation is "normal" in the sense that it occurs without environmental stimulus as in the case of the phase variation of Salmonella, the antiserum selecting out the resistant immunological type, or whether it is the kind of induced variation produced by cultivation of a microorganism in the presence of homologous antibody to give rough variants.

The parallel between this and the successive relapses of certain types of trypanosomiasis is striking (Chap. 34). In the guinea pig infected with *Trypanosoma rhodesiense* recovery is due to the development of a specific trypanolysin, the relapse to the multiplication of serum-fast trypanosomes, second recovery to a second specific trypanolysin, and so on. In this case the serum-fast strains are immunologically distinct from one another. In *T. lewisi* infections in the rat, however, both a trypanolysin and a reproduction-inhibiting antibody, or ablastin, are formed; the inhibition of cell division does not, presumably, allow the development of serum-fast strains, and hence there are no relapses.

Diagnosis.[14] The diagnosis of relapsing fever is made by demonstration of the spirochetes in the blood during the onset of a relapse by direct microscopic examination or by animal inoculation. Either the usual blood smear or a thick film similar to that used for the detection of malarial parasites may be used. The films are air-dried and stained with Giemsa; Wright's stain is satisfactory for thin films. The spirochetes may also be found in fresh wet preparations by darkfield examination. Mice and/or rats may be inoculated intraperitoneally and blood smears made daily for as long as 2 weeks if the spirochetes are not found.

Immunological methods of diagnosis have not been satisfactory in the past but Stein[101] has prepared spirochete antigens by saponin hemolysis and washing of the spirochetes. Complement fixation, said to be preferable for diagnostic purposes, and agglutination with serums from infected persons or animals occur and are reported to be specific.

Epidemiology. The relapsing fever spirochetes are blood rather than tissue

parasites, and the infection is transmitted by blood-sucking insects, though rare cases of direct transmission may possibly occur. Two epidemiological types of the disease are to be distinguished, the one tick-borne and representing transmission from an animal reservoir of infection to man, and the other louse-borne infection spread from man to man.[100]

A tick vector was not recognized in this country until 1930, but the tick-borne disease is now known to be not uncommon and at the present time is the only type which occurs. Known endemic foci of infection exist in Arizona, California, Colorado, Idaho, Kansas, New Mexico, Nevada, Oklahoma, Oregon, and Texas and possibly also in Montana, Utah, and Washington. *Ornithodorus turicata* and *O. hermsi* are known to transmit the disease, and it seems probable that *O. parkeri* transmits it also, but final proof is lacking. *O. talaje* transmits the infection in tropical America but apparently not in the United States; *O. rudis* is regarded as the most important vector in the tropics in this hemisphere. The traditional vector, and the most common in West Africa, is *O. moubata*. There appears to be no specific relationship between any particular species of tick and any particular strain of spirochete.

Infection may persist for long periods in the tick; Francis has reported survival in starved ticks for as long as 5 years, and in re-fed ticks for 6½ years. Furthermore, the spirochete may be transmitted to the offspring, and even the third generation may be infected.[39] The spirochetes are present in the coxal fluid, saliva, and feces of infected ticks. Of these, the first two appear to be the more important in transmission of the infection to man. For example, *O. moubata*, an excellent vector, secretes coxal fluid copiously while feeding, thus making possible infection of the bite, while *O. hermsi*, which is not known to transmit the infection to man though it does so from rat to rat, does not pass coxal fluid while feeding. The relative importance of direct introduction of the spirochete into the bite by infected saliva is not altogether clear. It is probable that infection of the bite by contaminated feces occurs occasionally.

European relapsing fever is transmitted from man to man by the human body louse, *Pediculus vestimenti*, and has the epidemiological characteristics of louse-borne disease. In contrast to the tick-borne infection, the bite is not infected by the secretions of the lice; rather the infected louse must be crushed on the skin and the spirochetes present in the body fluids contaminate the bite. The infection is transmitted to the egg, and there is some evidence that the disease may be produced by crushed eggs laid by infected lice.

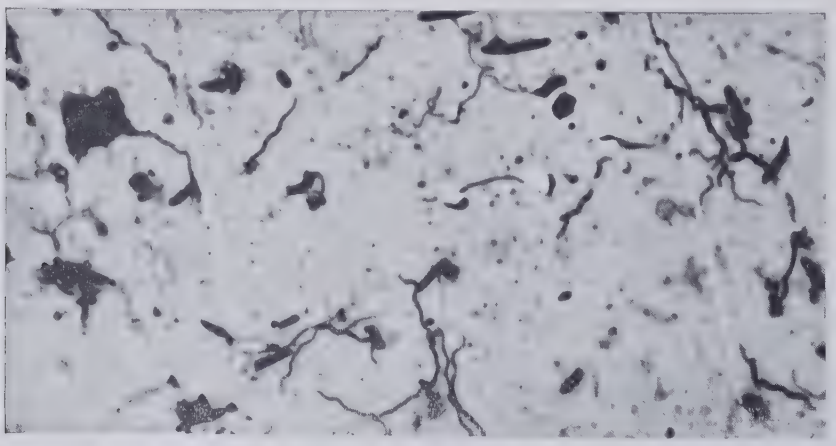


Figure 226. Smear from Vincent's angina showing the characteristic mixture of spirochetes (*Borrelia vincentii*) and fusiform bacilli. Fuchsin; × 1250. (P. E. Harrison.)

It is possible that the fingers may become contaminated by crushing infected lice and transmit the infection by scratching. The bedbug may also serve as a mechanical carrier of the infection.

Spirochetosis of fowls is transmitted by a tick, *Argas persicus,* which infects fowls in the warmer parts of the world, and, as in human relapsing fever, the spirochete is transmitted through the egg of the tick to the offspring.

SPIROCHETES OF THE MOUTH

As described elsewhere (Chap. 27), spirochetes are a part of the normal flora of the mouth which, together with other microorganisms, may increase in numbers in ulcerative conditions of the mucous membranes and in peridontal disease. These forms are usually regarded as species of Treponema, *viz., T. microdentium,* etc. In the ulceromembranous stomatitis and angina known as Vincent's disease, Vincent's angina, or trench mouth, these forms, together with fusiform bacilli of the Bacteroides group, increase in numbers and predominate in stained smears from the necrotic lesion. Other bacteria, cocci, and bacilli are present also, and the typical microscopic picture is illustrated in the accompanying figure.

The spirochete found in such preparations is known as *B. vincentii* or *T. vincentii* for the two genera cannot be separated on morphological grounds alone (see below). Whether this microorganism constitutes a distinct species of spirochete and plays any part in the development of the pathological process is questionable. It seems clear that the oral spirochetes are not pathogenic in the sense of being specific etiological agents of disease but, together with other microorganisms, may act synergistically in the accentuation, if not the initiation, of such suppurative infections.[12]

TREPONEMA AND THE TREPONEMATOSES[43, 112]

Spirochetes of the genus Treponema are morphologically indistinguishable from Borrelia, but the pathogenic forms are set apart from the relapsing fever spirochetes by the kinds of diseases which they cause. The pathogenic forms are *Treponema pallidum,* the causative agent of syphilis; *T. pertenue,* the causative agent of yaws; *T. carateum,* the causative agent of pinta; and *Treponema cuniculi,* the causative agent of rabbit syphilis. *T. microdentium* and *T. macrodentium* are nonpathogenic, and a part of the normal oral flora as indicated above, but are, in combination with fusiform bacilli, associated with ulcerative disease of the mouth, and this combination of microorganisms has also been found in certain types of tropical ulcer.[110]

The pathogenic species of Treponema make up a group of interrelated microorganisms, separable by differences in the kinds of disease they produce. Conversely, the infections with these microorganisms, known collectively as the treponematoses, are at the same time both similar, as with respect to the immune response, and differentiable by the character of the clinical disease. The treponematoses fall into three, or four, groups, *viz.,* syphilis which is divided into the venereal and nonvenereal forms, yaws or tropical syphilis, and pinta or pintid yaws. The mode of transmission is thought by some to relate to differences in the diseases, *i.e.,* the lack of systemic cardiovascular and neurological involvement in the nonvenereal forms.

The origin of treponemal infection has been a matter of absorbing and perennial interest. It is believed by some that an ancestral type of treponema, subjected to varying climatic, social, ethnological, economic, and other factors may

have differentiated into the several present forms of the microorganism, differing in their mode of transmission and in their pathogenicity with consequent variability in the human disease.[25, 116]

A disease similar to syphilis was present on the China coast in ancient times, the Biblical plague of Moab is thought to be syphilis, and syphilitic infection apparently occurred in Eastern Europe prior to the fifteenth century. Veneral syphilis, or morbus gallicus,[54] occurred in epidemic form in Europe after the return of Columbus from the Americas, and it is believed by some that this form of treponematosis was introduced from the Western Hemisphere.[48] There is evidence that syphilitic infection may persist in nonvenereal form following epidemics of venereal syphilis. The Scottish yaws or "sibbens" occurring in the seventeenth century after the time of Cromwell,[63, 82] "radesyge" in Norway, and "spirocolon" in Greece and Russia in the nineteenth century appear to be nonvenereal syphilis derived in this way. With improving economic and sociological conditions, the disease tends to die out—the last case of sibbens, for example, was reported more than 100 years ago—but it persists at the present time in the Near East, in Africa, and in Central Europe, and occurred in epidemic form in Chicago in 1949.

The earliest description of a yaws-like disease appeared in 1558 as occurring in Brazil, and the disease as known today was identified in the seventeenth century in the West Indies and in Brazil in the eighteenth century; it is now known to be ubiquitous in tropical regions. Pinta is more recently identified as a treponematosis occurring in Mexico, Cuba, and adjacent areas; the spirochetal etiology was established in 1938 by Cuban workers on the basis of earlier studies by Herrejon; formerly it had been thought to be a fungous infection.

While the differences among these diseases are felt by some to require considering them as separate entities,[77] the differences are not much greater than those between venereal syphilis in the Middle Ages and present-day venereal syphilis. It is believed by many that the similarities are more striking than the differences and these, together with other considerations, have led to a "unitarian" view of the treponemal infections in which the several clinical manifestations are considered to be variations on a central theme.

Cultivation. Schereschewsky reported the cultivation of *T. pallidum* in 1909, but his cultures appear to have been contaminated with other microorganisms. Noguchi later reported the growth of several strains of Treponema in pure culture under strictly anaerobic conditions in a serum water medium containing a piece of sterile fresh rabbit kidney or testicle. A number of such strains of spirochetes have been similarly isolated and may be grown in a heart infusion broth containing glycose, cysteine, and filtrate of coagulated plasma,[90] the best known of which are the Reiter, Nichols, Kroo, and Kazan strains. In other instances the spirochetes may survive for extended periods,[114] but are not actually cultivated. The Reiter strain, although useful in serological studies,[92] was originally virulent for rabbits when isolated by Wassermann and Ficker in 1922, later became completely avirulent but seems to have retained its antigenicity. The Nichols strain has retained virulence not only for the rabbit but also for man as shown by inoculation of human volunteers.[65] In that study 57 spirochetes infected half of 8 nonsyphilitic individuals, and the ID_{50} for the rabbit was 23 spirochetes.

The Treponemas are extremely fragile microorganisms and die out quickly outside the body. They are destroyed by soap and water or by drying, and are unusually susceptible to heat; suspensions of infected rabbit testicle are sterilized at 41.5° C. in 1 hour and in 2 hours at 41° C., but the cultivable strains may remain viable in culture mediums kept at 37° C. for as long as a year. The viability of *T. pallidum* is of interest in connection with the possibility of transmission of syphilis by infected blood; although occasional transmission has occurred in this way, the risk is not great

with blood from blood banks, for the spirochete dies out in 3 or 4 days at refrigerator temperature and quite rapidly in lyophilized plasma.

Pathogenicity for Animals. Syphilis may be transmitted to anthropoid apes, such as the chimpanzee and gibbon, and, with less certainty, to monkeys, as shown by Metchnikoff and Roux in 1903. Scarification of the genitals or eyebrow results in development of a primary chancre followed in a few weeks by the appearance of lesions of secondary syphilis.

Rabbits may be infected by inoculation of the anterior chamber of the eye. The local wound heals, but in 4 to 6 weeks pericorneal congestion develops which is followed by pannus and keratitis; then retrogression and healing occur. The entire process may take many weeks to complete. Intratesticular inoculation or implantation produces orchitis and intrascrotal inoculation a primary chancre; generalized lesions characteristic of secondary syphilis follow. A generalized infection may be produced by intravenous inoculation of very young rabbits. The spirochetes remain alive indefinitely in the lymphatics and may be obtained by excision of a popliteal gland. In rats and mice the infection is symptomless, but the spirochetes multiply in the tissues and the infection remains latent. Guinea pigs react similarly but may develop a local reaction following intracutaneous inoculation in the perineal fold.

The rabbit has been the experimental animal of choice, and the reaction to intradermal inoculation has allowed the differentiation of strains of Treponema with respect to pathogenicity. A small inoculum suffices to infect, and multiplication occurs exponentially after 24 to 48 hours, with the production of a mass of microorganisms and a visible lesion. With an inoculum of 500 spirochetes the incubation period is about 17 days; with an inoculum of 5000 microorganisms, 4 to 5 days, and the generation time is estimated to be about 30 hours.

In the initial stage hyaluronic acid is produced which appears to favor the multiplication of the microorganisms. In the second stage there is a mononuclear infiltration, and in the third stage an influx of polymorphonuclear leucocytes, and the infection may or may not involve the regional lymphatics. Three kinds of reaction in the rabbit, and also in the hamster, have been described by Turner and Hollander.[112] One of these, designated the Sh or syphilis type of reaction and given by *T. pallidum* from venereal syphilis, consists of an indurated lesion at the site of inoculation and lymphatic involvement. Samoan strains of *T. pertenue* produced only minimal local reaction and no infection of the lymphatics, designated the Yh reaction. An intermediate reaction, the Mh type, with pronounced local lesion and lymphatic infection, is given by some yaws strains and by spirochete strains from nonvenereal or endemic syphilis. Although it has been reported[62] that the rabbit may be infected with *T. carateum,* the infection has not been fitted into these types.

Immunity. The immune response in treponematosis is twofold. The antibody-like activity designated reagin has wide diagnostic utility (see below), characteristically reacts in complement fixation and flocculation tests with nonspecific cardiolipid prepared from normal tissues, and its titer is correlated with the status of the clinical disease, disappearing when cure by chemotherapy is effected. The other is an antibody response to antigens present in the spirochetes, demonstrable with spirochetal antigens as immobilization and other phenomena (see below). It is generally believed that there are three kinds of antigens present in the spirochetes, a thermolabile protein antigen, a thermostable polysaccharide antigen, and a lipoidal antigen similar to the material present in cardiolipin preparations.[30, 86] There appears, however, to be no immunological difference between the spirochetes of venereal syphilis, nonvenereal syphilis, yaws, and pinta, and all of these diseases give positive reactions to the same serodiagnostic tests.

SYPHILIS (TREPONEMA PALLIDUM)[10]

Syphilis, by far the most intensively studied of the treponematoses, occurs, as indicated above, in two forms. One is venereal syphilis and is the best known form, and the other is nonvenereal or endemic syphilis. The two are epidemiologically distinct in that transmission is almost always sexual in the first instance, but in the second the infection is transmitted both by direct nonsexual contact and indirectly by the common use of eating and drinking utensils among children and adolescents. Consistent with this, the primary lesion is usually on the genitalia in venereal syphilis, but the first lesion occurs as oral mucous patches in the childhood disease. Here venereal syphilis will be considered in more detail, and the nonvenereal form and the other treponematoses of man will be considered in relation to it.

The prevalence of syphilis is not known definitely; the estimate that 10 per cent of adults in the United States will give positive Wassermann tests is probably fairly accurate. It is also estimated that between 1 and 2 per cent of the children in this country have congenital syphilis. The mortality from syphilis is also not known. The death rate of 13 to 14 per 100,000 is misleading, for deaths reported as due to other causes are frequently due to syphilis; all cases of general paresis, locomotor ataxia, and many cases of apoplexy and aortic aneurysm are, for example, due to syphilitic infection, and the disease is a contributory cause in deaths from other diseases. Studies on the incidence of syphilis in autopsies on adults have given rates of from 2.6 per cent to 29.5 per cent; the average in 146,761 autopsies performed from 1896 to 1938 was 5.45 per cent. In this country the prevalence is much higher in the Negro than in the white; tests of the first two million selectees under the Selective Service Act showed 2 per cent in white and 22 per cent in Negroes. There is considerable variation in the ratio from one part of the country to another.

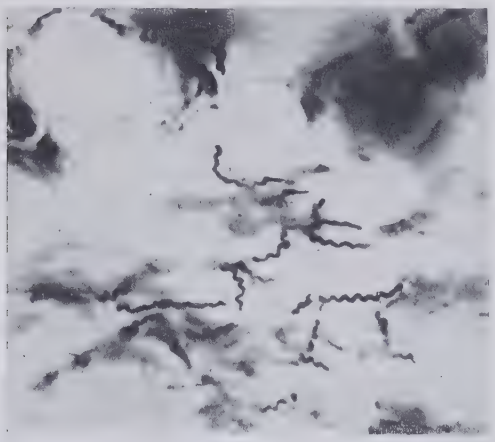


Figure 227. *Treponema pallidum* in stained (silver impregnation) smear; × 2000. (Kral.)

The number of reported cases continued to fall in this country until 1955, remained steady until 1958, and then increased. It is estimated that the reservoir of untreated syphilis is 1,200,000, and that the annual increase is about 60,000. There are currently about 33,000 paretics in institutions.[18]

The Disease in Man. A disease of protean manifestations, syphilis in man assumes several relatively well-defined clinical stages. The ordinary course of the disease includes the successive appearance of the so-called primary, secondary, and tertiary stages; congenital syphilis is best regarded as a separate subdivision.

Primary stage. Following the penetration of the skin or mucous membranes, the spirochetes rapidly invade the tissues; it has been found that the inguinal lymphatics of rabbits become infective 30 minutes after cutaneous inoculation of the scrotum. The initial primary lesion appears 10 to 30 days following infection. The typical hunterian chancre (so called because it was described accurately and in detail by John Hunter) is an indolent, indurated ulcer, single and painless, and not infrequently atypical and trifling in appearance. The presence of the spirochetes in the chancre serum may be demonstrated by darkfield examination and constitutes the diagnostic method in early primary syphilis, for a positive Wassermann reaction (see below) does not develop

until 2 or 3 weeks after infection. Clinically, then, syphilis in the primary stage is a localized infection, but bacteriologically it is a generalized infection, and spirochetes are often present in the blood stream.

Secondary stage. In the secondary stage the generalized nature of the infection is apparent. Bones, joints, eyes, and other organs are invaded with the appearance of constitutional symptoms, cutaneous lesions, enlargement of the lymph glands, pains in the joints, and the like. Evidences of the development of this secondary stage are usually apparent 4 to 8 weeks after the appearance of the chancre but may be delayed for a year or more. Spirochetes are present in all the lesions and may be found in the blood stream.

Tertiary stage. The disease passes insensibly into the tertiary stage and may persist for years. Ulcerating necrotic lesions of the skin and gummata of the internal organs are common, and the clinical manifestations of infection are so highly diverse that there is no characteristic picture. Spirochetes are present in the tertiary lesions but only in small numbers.

Neurosyphilis. Spirochetal invasion of the central nervous system may occur independently of the so-called stages and as soon as the early secondary stage. Generally, however, neurosyphilis is a manifestation of late tertiary infection and may appear years after the initial infection, and tabes dorsalis, general paresis, and other evidences of the invasion are sometimes termed quaternary or parasyphilitic affections. The spirochetes may be found in the cord in tabes dorsalis and in syphilitic brains in the cortex, in the pia and vessel sheaths. General paresis is, then, a diffuse spirochetosis of the brain involving the cortical layers chiefly, and the presence of the spirochetes in the parenchyma explains much of the histopathological picture and the nervous symptoms of the affection.

Latent syphilis.[29] In some instances the primary lesion is not noticed and the secondary manifestations may be slight, and with their subsidence there is no clinical evidence of disease and the infection remains latent within the tissues. In other instances the latent condition develops after primary and secondary symptoms of usual severity. Such clinically latent infections may, perhaps, be regarded as the maintenance of a biological balance between the host and the parasite. Latent infections may, of course, develop clinical manifestations at a later time.

Congenital syphilis. The fetus is readily infected *in utero* by spirochetes passing through the placenta. The infection may result in a cessation of development and abortion, or the fetus may go to term and be born dead. In those who survive, the disease is generalized from the beginning, and the lesions are those of tertiary syphilis. Spirochetes may be demonstrated in great numbers in the tissues and organs affected. Third generation syphilis has been reported but is very rare.[70]

Immunity. At the present time syphilis is not so severe a disease in man as it was in the early sixteenth century. Whether this is an expression of an adaptive response on the part of man with the development of a low degree of natural immunity, or a similar response on the part of the parasite manifested as a decrease in virulence, is not known; possibly both may have occurred.

The specific immune response to syphilitic infection is not well understood. There is a marked apparent insusceptibility to reinfection that is illustrated by the fact that a second chancre may be produced by reinfection prior to the appearance of the first chancre, but after the first chancre has appeared further reinfection does not produce an initial lesion. It is commonly stated that man, once infected, is refractory to reinfection and that reinfection occurs only very rarely. In other words, there is an infection-immunity or immunity to superinfection superficially similar to that observed in tuberculosis. This tends to be substantiated by inoculation studies in human volunteers[65] referred

to above in which 100,000 spirochetes failed to infect individuals having untreated latent syphilis, 10 of 26 persons who had been treated for proved or presumed latent syphilis, while the ID_{50} for nonsyphilitic individuals was 57 spirochetes.

A certain degree of immunity is developed, especially later in the disease. Protective antibody is demonstrable, and in the rabbit a partial immunity, produced by infection and cure with penicillin after varying times, inhibits to a certain extent dissemination of infection following subsequent inoculation. In addition, the spirochetes are immobilized and killed in syphilitic serum in the presence of complement. An *in vitro* immobilization test has been devised for the demonstration of this activity (see below), but it is not clear to what extent it is associated with effective immunity.

Serodiagnosis.[106] As indicated above, the immune response is of two kinds, the appearance of Wassermann antibody, or reagin, and of antibody to antigenic substances present in the spirochete. The former has been widely used for diagnostic purposes, while the latter response is immunologically specific, but its assay is as yet too complex for general application.

A complement fixation test was proposed by Wassermann and his coworkers in 1906 as an immunologically specific reaction, using a watery extract of syphilitic fetus as the antigen. It was soon found, however, that syphilitic serums fix complement in the presence of an extract of normal tissue.

Not long after the development of the Wassermann test it was observed that syphilitic serums produce a flocculation when mixed with Wassermann antigen. The reacting substance present in the antigen preparations occurs in various tissues, but heart (beef) muscle extracts are the most satisfactory. It is soluble in alcohol and insoluble in acetone, and its activity is considerably increased by the addition of cholesterol. Activity of these extracts is due to a phospholipid, *cardiolipin,* which is precipitated from the initial alcoholic extract with cadmium chloride, freed from lecithin, and fractionated by precipitation from ether with acetone and alcohol. Anticomplementary activity of the preparation is removed by the addition of lecithin,[61] and complement is not fixed unless cholesterol is added. The function of the lecithin is not clear, and it may function only as an emulsifying agent. The complete lecithin structure seems to be required, and dimyristoyl lecithin may be substituted for natural lecithin.[3] According to Pangborn,[80] who described this substance in 1941, it is a complex phosphatidic acid. A similar product of vegetable origin (wheat germ) sitolipin, has been prepared. The activity appears to be a property of similar substances varying in their fatty acids; lauric and linoleic acids have been found to be present, together with a third component.[89]

Types of tests. Probably no serological reactions have been as intensively studied as the complement fixation and precipitation tests for syphilis, and the techniques have been modified and refined by many workers to increase sensitivity and specificity. At the present time two complement fixation tests, the Kolmer test and the Venereal Disease Research Laboratory, or VDRL, test, and four precipitation tests, the Kahn test, the Kline test, the Hinton test, and the Eagle precipitation test, are used. In addition to these, more sensitive precipitation tests have been devised by Kahn and by Kline and are known as the Kahn presumptive test and the Kline exclusion test; they are apt to give false positive reactions but are regarded by some workers as having considerable utility as screening tests. A rapid unheated serum reagin, USR, is used also for presumptive purposes.[115]

The complement fixation test may also be quantitative, *i.e.,* the reagin present in the syphilitic serum may be titrated. For ordinary diagnosis this is unimportant since it is not known whether the reagin titer has any significance, but it is useful in following therapy and in the diagnosis of congenital syphilis in infants. In the latter case reagin is passively transferred from

the maternal circulation; if the infant is not infected the reagin titer drops slowly, but a gradually increasing titer is indicative of infection.

Specific tests. Any serological test making use of spirochetal antigen, and thus having immunological specificity in the conventional sense, should be much more precise. An antigen may be prepared from *T. pallidum* by making an extract of infected rabbit testicular tissue with citrate, acetone and desoxycholate for use in the *T. pallidum* complement-fixation, or TPCF, test.[66, 85] The antigen may also be obtained by differential centrifugation of *T. pallidum* from infected rabbit tissue, cryolysis, and ammonium sulfate precipitation to give the *T. pallidum* cryolysis complement fixation, or TPCP, test. The cultivable Reiter strain contains antigens in common with *T. pallidum* as demonstrable by absorption tests[19] and may also be used as a source of antigen in the Reiter protein complement fixation, or RPCF, test.[98]

The treponema immobilization test (TPI), in which the living motile spirochetes are specifically immobilized in the presence of antibody, complement, and thioglycollate (the last to maintain anaerobic conditions), has been devised.[78, 79, 109] It has been widely and critically tested and found to be highly specific.[15] The fluorescent antibody technique has also been applied to the serodiagnosis of syphilis, using both the direct and indirect methods[33, 34] to give a fluorescent treponemal antibody, or FTA, test. The test is highly sensitive and correlates well with other serological methods.[37, 75]

The wide variety of serodiagnostic tests is an expression of a continuing search for a completely reliable test, but it is probable that no single test will suffice in the face of complications of therapy, biological false positives and negatives, etc. Some combination of tests may be required as indicated by circumstances (see below); one such combination which has been suggested[20] is that of the VDRL slide test, the RPCF test, and the TPI test.

The effect of therapy. The majority of individuals undergoing therapy become serologically negative to the cardiolipin type of test in time, and this response is usually taken as an indication of the efficacy of the treatment. The time required to attain negativity is variable and depends upon the individual, the stage of the disease, the course of therapy, especially if continuous or intermittent, and the sensitivity of the serological test used. No general statement of any precision is possible, therefore, but it is usually said that the majority of persons with primary or early secondary syphilis become negative after one or two courses of arsenical or continuous treatment for 5 to 7 weeks. Some, however, become negative with only one or two injections of drug, while others may remain positive for months, and a certain proportion remain positive indefinitely. The last is spoken of as Wassermann-fast, reagin-fast or seroresistant syphilis.[51] Slightly over 10 per cent of infections are in this group. Two explanations are usually offered for seroresistant syphilis, one that persistent foci of infection remain, and the other that the continued presence of reagin is evidence of a definite immune response.

Specificity and false reactions. False negative reactions are in part a technical matter; serological tests in general use give 80 to 90 per cent positive reactions with known serums, and any test which gives more than 80 per cent positives is regarded as satisfactory. The presence of insufficient reagin accounts for many false negative reactions and occurs in several stages of the disease, especially in early primary syphilis (a positive reaction usually does not develop until 2 to 3 weeks after infection) and late syphilis which is latent or localized. Tabes, for example, gives about 40 per cent negative reactions. Negative reactions may also be a result of the presence of antibody to the Forssman antigen, acquired for example by prior infection with microorganisms containing the antigen, such as certain strains of pneumococci, in the serum tested. If such antibody is present to reasonably

high titer, the sheep cells of the indicator system are lysed in the presence of complement. This may be avoided by a preliminary absorption of the serum with sheep cells.

False positive reactions may be a result of technical error or may be biological in nature. A certain proportion of nonsyphilitic individuals suffering from other infections give positive serological tests for syphilis. All cases of yaws are Wassermann-positive and it is reported that 4 to 10 per cent of cases of malaria are positive. Brucella infection has also been reported[21] to be associated with false positive reactions. Febrile disease of one sort or another occasionally produces isolated or repeated positive reactions.

False reactions occur less commonly with the specific tests using treponema or treponemal antigen, and these have their greatest utility when the more readily performed slide precipitation and other kinds of nonspecific reactions give equivocal results.[24, 38] The treponemal tests are subject to error also, but these may be technical in nature.[93]

The nature of the response to infection with the spirochete that results in the appearance of reagin is not clear.[56] Its lack of immunological specificity would seem to rule out the usual type of antibody response, but if it is to be regarded as antibody, the facile disappearance of reagin during therapy rather than seroresistant syphilis requires explanation. It is possible that reagin represents iso-antibody that is detectable only because it is being produced more rapidly than it can be removed by union with antigen.

Chemotherapy. The chemotherapy of syphilis, like that of tuberculosis, has been of great interest for many years. The long-term treatment with arsenicals and bismuth was highly effective for the relatively small number of patients who did not default. For example, of 1340 cases treated over a period of 4 years and followed for an average of 11 years, there were only nine failures of which four were reinfections.[35] This method of treatment is now a thing of the past although arsenicals and/or bismuth have been used in combination with penicillin.[23, 55]

Of the chemotherapeutic drugs now available, the sulfonamides are ineffective, but penicillin has been found to be highly effective. For example, in the research series of the U. S. Public Health Service, 1538 patients treated on various schedules were followed for 2 years. In this group 92.3 per cent of those having initially seronegative primary syphilis, 82.4 per cent of those with seropositive primary syphilis, and 78.3 per cent of those with secondary syphilis were cured. The relative efficiency of penicillin therapy and the adjuncts made use of are dependent upon the stage and nature of the disease.[28]

The broad-spectrum antibiotics, chloramphenicol and the tetracyclines are apparently almost as effective as penicillin in the chemotherapy of syphilis. As yet only relatively small numbers of patients have been treated with them, and their ultimate value can be determined only by studies carried out with large numbers of patients over a period of years. While as yet the incidence of penicillin-resistant spirochetes has not assumed practical importance, the occasional occurrence of penicillin-resistant strains coupled with individual sensitivity to this drug is indicative of the utility of alternative chemotherapeutic agents.

Nonvenereal Syphilis

Nonvenereal or endemic syphilis occurs in foci in many parts of the world under various local names. That occurring among the desert Arabs in the Near East is known as bejel,[53] that in Karanga people in Southern Rhodesia is njovera, dichuchwa is the disease found among the Bantus in Bechuanaland,[73] and the disease is known locally as siti in Gambia. It occurs in Southeast Asia and with some frequency in Eastern Europe, especially in Bosnia in Yugoslavia.[41] In the last area, prior to mass treatment under W.H.O. auspices, cases of the disease

made up as much as 5 per cent of the population.

The disease is an epidemiological rather than clinical entity, occurring, as indicated earlier, largely in children and within families. There appears to be little or no basis for separating the diseases occurring in widely separated foci from one another or from venereal syphilis. Clinical differences between venereal and nonvenereal syphilis, *viz.*, the relative rarity of primary lesions and congenital manifestations in the latter, seem to be a consequence of the mode of transmission and age distribution of the disease rather than of differences in the pathogenicity of the causative microorganism. There is, however, some uncertainty as to the frequency of cardiovascular and neurological involvement; it seems to be relatively rare, but not unknown, in nonvenereal syphilis, but the nonvenereal form of the disease may occur mixed with venereal syphilis.

The first manifestations of the infection are most commonly mucous patches in the mouth, and skin lesions including anogenital condylomata occur also. These lesions show the presence of spirochetes on darkfield examination, as do the lesions found on the nipples of mothers infected by children. In most individuals latency occurs after the early stages, with subsequent development of tegumentary lesions, including gummatous destruction of the skeleton, which are indistinguishable from those of venereal syphilis. The causative microorganism is *T. pallidum,* and the disease is effectively treated with repository penicillin.

YAWS (TREPONEMA PERTENUE)[105, 111]

Yaws, or frambesia tropica, is a disease occurring in tropical countries and is common in Equatorial Africa, the tropical regions of the Far East though rare in India, the West Indies, and tropical America. The incidence may be high, 5 to 20 per cent, and the disease is a major public health problem in Haiti. It is prevalent in rural rather than urban areas and associated with low economic status, *i.e.*, the wearing of clothing appears to protect. Primary infection is more common in children than in adults. The causative agent is *T. pertenue* which was described by Castellani in 1905.

The spirochete is found in the serous exudate of the cutaneous lesions and in the lymphatics. It may be demonstrated in Giemsa-stained smears or by darkfield examination of fresh preparations and is morphologically indistinguishable from *T. pallidum.*

The disease in man[45] is characterized by a papular eruption. The commonest site of infection, perhaps in 75 per cent, is the lower leg and foot. A general malaise precedes the appearance of the initial lesion which is practically always extragenital and takes the form of a single papule or a small group of them. The papule enlarges to a diameter of 3 to 4 mm., the thickened epidermis cracks, and the fungoid mass beneath exudes a seropurulent fluid. The mass enlarges to 3 to 5 cm. in diameter and the lesion is termed a yaw. When the infection occurs on the sole of the foot, it is known as crab yaws or wet crab yaws. It eventually dries, leaving only a scar. Six weeks to 3 months after the primary lesion appears, a secondary eruption occurs which is similarly preceded by a general malaise. The lesions are of the same general character as the primary lesion, appearing on the extremities, neck, and at the juncture of the skin and mucous membrane about the nose, mouth, and anus. Tertiary lesions similar to those of syphilis are said to be rare, but recent work suggests that they may be more common than has been supposed. The Wassermann reaction is positive, and in certain stages, as in late secondary eruption, differential diagnosis may be very difficult.[47]

Yaws may be produced by the inoculation of monkeys and rabbits. The infection in rabbits with *T. pertenue* is similar to that with *T. pallidum,* but relatively few spirochetes are found in

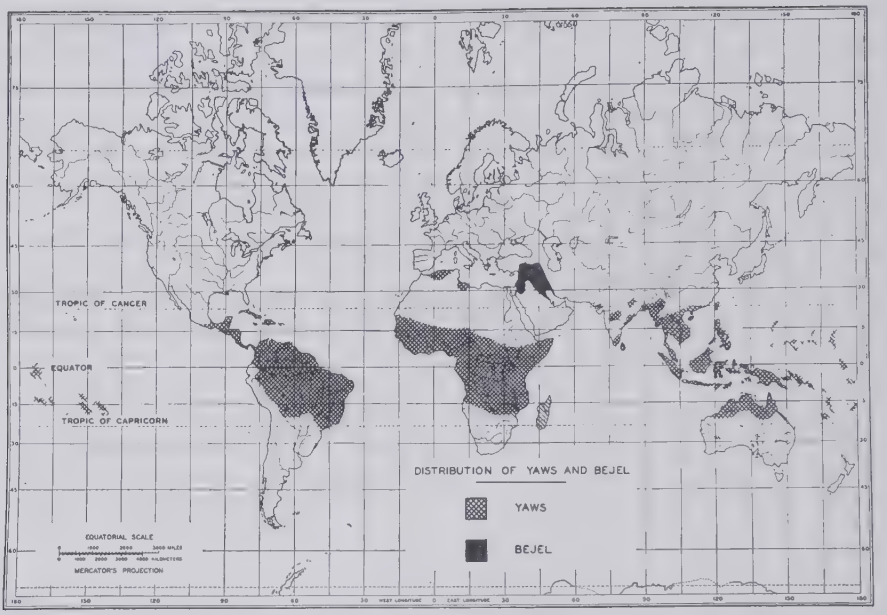


Figure 228. The world-wide distribution of yaws and bejel. Redrawn from map prepared by Army Medical Intelligence. Based on Goode *Base Map* No. 201M. By permission of the University of Chicago Press.

the lesions, multiplication is arrested early, and the inflammatory reaction is slight.

The disease is seldom venereal, and the commonest mode of transmission is contact between individuals.[44, 46] Trauma of the skin usually precedes infection, and the ability of the spirochete to penetrate the intact skin is questionable. Biting flies may also transmit the disease and in Jamaica the small fly, *Hippelates pallipes*, feeds upon the serous exudate in huge numbers, the spirochete surviving for some hours in the diverticulum of the fly and possibly being regurgitated when the fly feeds upon a skin abrasion.

Yaws may be successfully treated with penicillin, and there is evidence that chloramphenicol and tetracyclines may also be effective. The change to seronegativity is usually not as rapid as in effectively treated syphilis, possibly because the disease is usually of longer standing.

The relationship of yaws to syphilis is very close, the points of difference being the nonvenereal transmission of yaws, the differences between the yaw and the indurated hunterian chancre, and the relative infrequency of visceral and tertiary lesions. Yaws is regarded by many as a tropical type of syphilis, differing, not necessarily qualitatively, from endemic syphilis as a result of environmental factors.[42] There is some evidence of a cross-immunity between syphilis and yaws in that the two diseases tend to be mutually exclusive, but precise evidence is lacking.

PINTA (TREPONEMA CARATEUM)[22, 52, 68]

Pinta, known in Mexico as *mal de pinto* and in Colombia as *carate*, is clinically a skin disease in which dyschromic changes occur in patches which may be discrete and small or large and confluent, and of a gray, bluish gray, or pinkish color and eventually white. It is especially prevalent in Mexico, where 11 per cent of over two million persons examined had the disease, and in Colombia, where as much as 4 per cent of the population in some districts are affected. It also occurs in Cuba and the West Indian Islands and in Central America and tropical South America. There is some question as to whether the Mex-

ican and Cuban diseases are identical.[113] It is apparently confined to the Western Hemisphere for the most part, but cases have been reported from India.[40]

Etiology. For many years this disease has been of uncertain etiology though generally assumed to be a fungous infection. It was suggested by Herrejon in 1927 that the disease is caused by a spirochete, but it was not until 1938 that a spirochete was first demonstrated by Armenteros and Triana in Cuba. The observation has been amply confirmed, and it is now established that pinta is caused by a spirochete which has been named *T. herrejoni* by the Cuban workers and *T. carateum* by Brumpt.

The spirochete may be found in material taken from early cutaneous lesions and in that aspirated from lymph glands. It stains with Giemsa and by the usual silver impregnation methods used for *T. pallidum* and may be found by darkfield examination of fresh material. Morphologically it is very similar to, perhaps indistinguishable from, the spirochete of syphilis. While it gives no cross reaction with *T. pallidum* in the immobilization test, the pattern of the universal serological reaction of Kahn is identical with that of syphilis. Attempts to cultivate it have failed, but it has been reported that the rabbit may be infected. The experimental disease in human volunteers has been studied in considerable detail, however, especially by Leon y Blanco.

The Disease in Man. In the experimental infection the incubation period is from 7 to 20 days, the initial lesion being a papule which appears at the site of inoculation. This papule spreads peripherally to form a squamous, erythematous patch reaching a diameter of 1 cm. in 4 or 5 weeks. It continues to spread, varying considerably in appearance, and may be lichenoid or psoriaform. There is less inflammatory reaction than in either syphilis or yaws.[49] Leon regards this as the primary stage. In about 5 months the secondary stage begins with the appearance of secondary lesions about the initial lesions and elsewhere on the body. Progressive hyperpigmentation occurs, and depigmentation, resulting in varied hues, follows in the third stage; keratosis and superficial atrophoderma become apparent. Hyperkeratosis on the plantar surfaces occurs in Cuba but is seldom observed in Mexico. The Wassermann reaction is almost always negative in the primary stage, positive in something over half the cases in the secondary stage, and practically always positive in the tertiary stage. Correspondingly, superinfection is readily produced in the first stage but not in the third stage. Syphilitic individuals may be infected without difficulty. Pinta may be successfully treated with penicillin.

Transmission.[99] Experimental human infections have shown that serous fluid from the early lesion is highly infectious when placed on an abraded area on the skin, and the spirochete may be demonstrated in the discharge from the fissures in plantar hyperkeratosis. Contact infection would appear to be possible, but general opinion is that it is not an important method of spread. Simulium flies have been suspected of transmitting the disease, but as yet an insect vector has not been demonstrated.

LEPTOSPIRA AND LEPTOSPIROSIS[4, 5, 16, 107]

Leptospirosis, or infection with leptospira, is a not uncommon and widely distributed affection. Several species of leptospira are involved, and the infections differ somewhat in their clinical manifestations.

Morphology. The leptospiras differ from Treponema and Borrelia species in that their spirals are very fine and close, one or both ends of the cell may be hooked, and the individual cells are smaller, not more than 0.3 μ in breadth

and generally 6 to 10 μ in length, although exceptionally forms as long as 25 to 30 μ may be observed. The protoplasm is spirally wound about a delicate but firm, elastic, smooth axial filament enclosed within a well-defined cell wall, which is clearly shown in electron micrographs.[97, 103] Like the finer bacterial flagella, the axial filament is not seen in the darkfield or ordinary stained preparations but may be demonstrated by silver impregnation flagella stains. The periplast is thick and almost transparent and appears in the darkfield as a narrow, clear zone or halo and in certain stained preparations as a grayish or unstained halo. The mechanism of locomotion is more complex than in the treponemas; the hooked ends of the axial filament are probably involved, and when the bow-like axial filament is alternately straightened and relaxed by rhythmic contractions of the protoplasmic spirals the cell rotates.

The leptospiras are readily filterable through Berkefeld V and N candles because of their small breadth and active motility, and filtration is sometimes used to separate them from bacteria. Leptospiras are usually not filterable by ordinary suction methods but grow through the pores of Berkefeld candles with some facility.

Cultivation. The leptospiras are the most readily cultivable of the spirochetes. They may be grown in a liquid medium consisting of 1 per cent peptone in phosphate-buffered Ringer solution to which sterile rabbit serum is added in a final dilution of 1:40, or in a semisolid medium consisting of meat extract agar enriched by the addition of sterile serum. In the isolation of leptospira from the blood, the inoculum is in the proportion of 0.03 ml. of blood to 15 ml. of medium, and the culture is incubated for as long as 28 days before discarding. The blood presumably satisfies the nutritive requirement for iron.[36] One of the most readily prepared liquid mediums for the cultivation of leptospiras is that described by Cox,[26] consisting of 0.2 per cent tryptose-phosphate broth enriched by the addition of normal rabbit serum to 10 per cent. In the isolation of leptospira from water, agar medium and blood may be added to Berkefeld V and N filtrates. The leptospiras are all obligate aerobes. Those tested do not ferment sugars and are indifferent to the presence of carbohydrates in the medium.

Colonial growth of leptospiras on solid medium has been described by Cox and Larson[27] on tryptose-phosphate-normal rabbit serum medium enriched with rab-

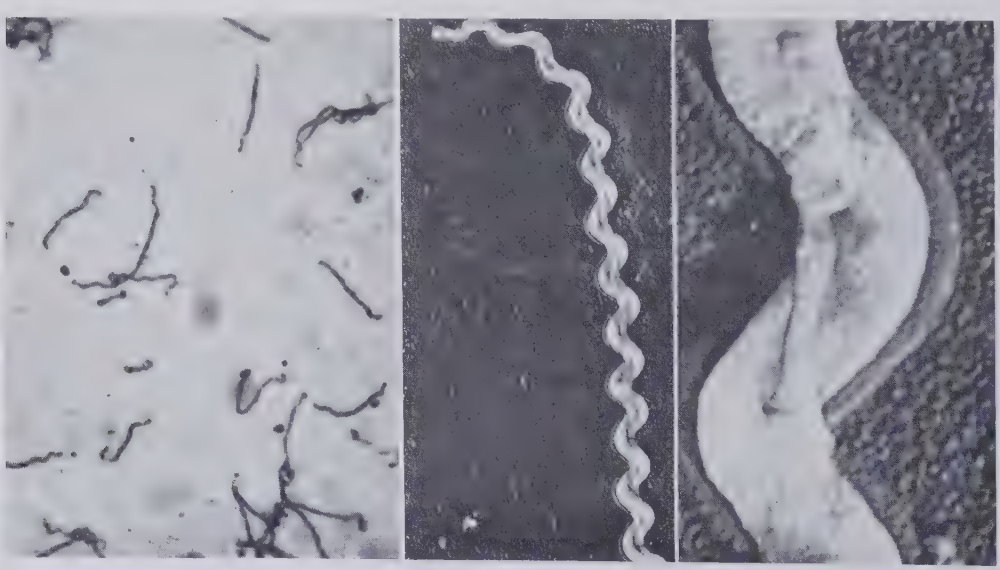


Figure 229. Leptospira morphology. *Left,* conventional stained smear of *Lept. pomona. Center* and *right,* electron micrographs of chromium-shadowed spray preparations of *Lept. icterohemorrhagiae. Center,* × 12,500; *right,* × 125,000. (Simpson and White.[97])

bit hemoglobin and solidified with 1 per cent agar. Two types of colonies were observed, a small relatively opaque type and a larger translucent form ranging in size from 1 to 4 mm. in diameter.

Cultures are maintained[57] in liquid or soft agar medium which may be covered with a thin layer of paraffin oil to retard evaporation. The cultures are incubated at 28 to 30° C. for 1 to 2 weeks during which leptospiral growth appears as a band of turbidity a few mm. below the surface. Such cultures remain viable for 5 to 6 months when stored at room temperature, and should be transferred as 0.5 ml. of inoculum every 3 to 4 months. For serological purposes the cultures should be young, 1 to 3 weeks old. It has been reported[6] that leptospira may be kept for as long as two years in lyophilized form when stored at refrigerator temperatures.

Antigenic Structure.[8, 118] The leptospiras are excellent antigens, and agglutinating, lytic, and complement-fixing antibody is produced to high titer and persists in the case of recovered individuals, man or lower animal, to allow diagnosis of past infection. The several species or types of leptospira are differentiable and characterized on an immunological basis, and about 60 serotypes have been established with new serotypes being added from time to time. The serotypes have been given place or descriptive names, implying that they are different species, but no formal classification within this group is firmly established. A collection of serotypes is maintained at Amsterdam in Europe and at the Walter Reed Army Medical Center in this country.

These serotypes appear to be quite stable although antigenic changes can be produced by cultivation in antiserum.[81] There are minor cross reactions with some other microorganisms, including Shigella strains.[91] Immunity to experimental infection is largely type-specific with only occasional cross protection between serotypes.[96]

Pathogenicity. The pathogenic leptospiras are parasites of lower animals, including wild rodents and a variety of domestic animals of which the pig and dog, and in some areas cattle, are probably the most important quantitatively. Unlike Borrelia, they are not primarily blood parasites although they may be found in the blood as early as the first day and perhaps as late as the fourteenth day of the disease. The infection localizes in the tissues and organs, especially the kidneys, and the leptospiras are excreted in the urine.

Human infection is acquired from lower animals, by direct contact with infectious urine or water contaminated with infected urine or by direct contact with infected tissue. In the first instance the source of infection is most often rodents, and the leptospira penetrate the skin, and in the second, domestic animals, especially pigs, which serve as a source of infection for slaughterhouse workers, veterinarians, and herdsmen as in swineherd's disease in Switzerland.

Leptospiral infections in man are all closely similar and are characterized by a febrile reaction and jaundice which may be generalized or confined to the sclera. Weil's disease, or infectious jaundice, is the classic example of leptospirosis and has been known for many years. Other infections with leptospiras are much more common than has been realized. In many instances these are acquired through contact with mice and other infected rodents, and, since the infection often occurs in connection with working in cultivated fields and similar rural areas, the disease is variously known as swamp fever, field fever, harvest fever, and the like. Leptospirosis is given place names also, such as Mossman fever and Pomona fever. The etiology of these diseases is nonspecific in that there is little specific association of a given disease with leptospiral serotype.

Recovery from the disease is accompanied by the appearance of specific antibody and the development of an effective immunity. Prophylactic inoculation with leptospiral vaccines has been applied, with encouraging results, to groups of persons exposed to infection.[7]

The More Important Leptospiroses*

SEROTYPE	DISEASE IN MAN	IMPORTANT ANIMAL RESERVOIRS	INFECTIONS IN OTHER ANIMALS	GEOGRAPHICAL DISTRIBUTION
icterohemorrhagiae	Weil's Disease	*R. norvegicus*, other rodent species	dog, pig, cattle, horse	World-wide
canicola	Canicola fever	dog	pig, cattle	World-wide
pomona	Pomona fever, Swineherd's disease	pig, cattle, *Mus musculus*, *Apodemus agrarius*	dog, horse, opossum, raccoon, skunk, wildcat	World-wide
grippotyphosa	Mud fever, Schlammfieber, Field fever	*Microtus arvalis* *Evotomys glareolus* *Cricetus spp.* *Apodemus sylvaticus*	cattle, horse, dog, raccoon, goat	World-wide
autumnalis (*akiyami A*)	Hasami fever, Fort Bragg fever	*Microtus montebelloi* *Apodemus speciosus* *Bandicoote spp.*	dog, opossum, raccoon, cattle	S. E. Asia, Japan, U. S.
bataviae	Indonesian Weil's Disease, Rice-field fever	*R. norvegicus* *Micromys minutus* *R. rattus*	dog, cat	S. E. Asia, Europe, Africa, Japan
australis A (*ballico*)	Canefield fever	*R. conatus* *Apodemus flavicollis* *R. rattus culmorum*	dog, cattle, raccoon, opossum, hedgehog	Australia, U. S., Europe, S. E. Asia, Japan
australis B	Canefield fever	*R. rattus*, *Isoodon spp.*		Australia, S. E. Asia, Europe
sejroe	Feldfieber B	*Mus musculus* *Apodemus sylvaticus* *Apodemus agrarius* *Microtus spp.*	cattle (?), dog, pig	Europe, U.S. (?)
hebdomadis	Nanukayami Akiyami B, seven-day fever	*Microtus montebelloi*	dog, cattle	Japan
pyrogenes	Leptospirosis febrilis	*R. rattus* *R. brevicaudatus*	.....	S. E. Asia, Japan
ballum	.....	*Mus musculus* opossum	*R. norvegicus*, skunk, raccoon, wildcat, pig	U. S., Europe Israel
hyos (*mitis*)	Swineherd's disease	pig	cattle	U. S., Europe, Australia, South America, New Zealand

* Compiled by Colonel M. B. Starnes, Walter Reed Army Medical Center.

Laboratory Diagnosis.[48, 88, 117] The laboratory diagnosis of leptospirosis is based upon isolation of the infecting microorganisms and on the immune response to the infection. In man the bacteria may be isolated from the blood early in the disease by culture or animal inoculation; for the latter, young (150 gm.) guinea pigs or weanling (15 gm.) hamsters are the usual animals. The ex-

perimental infection may be substantiated by demonstration of the leptospira in the tissues, especially the kidney, by culture at autopsy, but in general animal inoculation gives a smaller proportion of positive results than does culture.

The immune response may be assayed as leptospiral agglutination (observed in the darkfield microscope), lysis in the presence of complement, or by complement fixation. The agglutination-lysis titers range from 1:400 to 1:100,000, and the complement-fixation titers from 1:32 to 1:256. A fourfold or greater rise in antibody titer in paired serums is regarded as diagnostic, but in an appreciable portion of cases (10 per cent?) demonstrable antibody is not present. The immune response in infection is ordinarily of sufficiently broad specificity that the serological identity of the infecting microorganism is not deducible from it, and for precise identification the leptospira must be cultured and typed serologically.

Chemotherapy. The chemotherapy of human leptospirosis has not been successful; penicillin, streptomycin, chloramphenicol, and tetracyclines have all been tested and found ineffective. However, chlortetracycline and streptomycin are reasonably effective in the dog, and streptomycin in the guinea pig.

WEIL'S DISEASE (INFECTIOUS JAUNDICE)

The causative agent of Weil's disease, *Leptospira icterohemorrhagiae,* was discovered in 1914 by Inada and Ito in Japan and was found in Germany the following year by Hübener and Reiter and by Uhlenhuth and Fromme. It was called *Spirochaeta icterogenes* by the German workers. The disease has been found all over the world; it is very common in Japan, less so in Europe where the greatest incidence is in the Netherlands and France, and is not uncommon in South America. It is probably not so rare in the United States as has been supposed.

Disease in Man. The incubation period is 6 to 12 days, and the high initial fever is followed by nausea and vomiting, epistaxis, headache, and muscular pains, and there may be moderately severe bronchitis. Jaundice is not always observed; it occurred in 40 to 60 per cent of the Dutch cases. Convalescence is slow, and weakness may persist for months.

The leptospira are distributed throughout the body in the first week of illness and may be demonstrated in the blood by guinea pig inoculation but rarely in blood smears. After the first week they are present in the urine and may continue to be so excreted for 4 to 5 weeks. There is some evidence that the case fatality is reduced by treatment with antiserum. In fatal cases death usually occurs during the second or third week, but occasionally as early as the end of the first week. The case fatality is variable; it has varied from 4.6 to 32 per cent in Japan and has been about 10 per cent in the Dutch cases and as high as 25 per cent in Scotland. At autopsy the leptospira are found in almost all the organs and tissues in those dying during the initial febrile stage, but if death occurs in the second week or later, they are rarely found elsewhere than in the kidneys. Acute renal failure is the commonest immediate cause of death.

Immunity. Recovery from the disease is accompanied by the development of a solid immunity, and a specific lysin is present in the blood which persists in detectable amounts for several years. Agglutinins appear in the convalescent stage, sometimes to very high titer, but the relation of demonstrable antibody and effective immunity is far from clear. Prophylactic inoculation has given encouraging results.

Transmission. The wild rat is the most important animal reservoir of infection, and Schüffner has reported that 40 per cent of tame rats raised for experimental purposes have been found to be infected in Holland. The infection may be detected by darkfield examination of urine and kidney emulsion, and preferably by the inoculation of young guinea pigs with kidney emulsion.

Larson has found that a fatal infection may be produced in young mice, 3 to 4 weeks old.

The proportion of infected rats is variable from one locality to another; in New York about 4 per cent of the rats have been found to be infected, in Japan 40 per cent, in England 30 per cent, in Rotterdam 7 to 40 per cent, and in Philadelphia about 10 per cent. The leptospira are discharged in the urine of infected rats and are transmitted to man via stagnant water contaminated with rat urine. Weil's disease is to some extent an occupational disease and occurs in coal miners, sewer workers, and others working in contact with contaminated water. The majority of the Dutch cases have been in swimmers, bargemen, and fishermen and those who by accident or intent fall into the canals. In one instance a water-borne epidemic occurred in which the water was presumably contaminated by rats from a neighboring sewer. The manner in which the spirochetes enter the body is not known, but very possibly it is through minute cuts and abrasions in the skin or via the alimentary tract.

CANINE LEPTOSPIROSIS

The role of dogs in the transmission of infectious jaundice has been of considerable interest. Dogs suffer from two forms of leptospirosis, the one an acute jaundice ("yellows") similar to acute Weil's disease in man and the other a non-jaundice type known as Stuttgart disease or canine typhus. *Lept. icterohemorrhagiae* may infect dogs and probably is responsible for the former type of disease, while a canine leptospira, *Lept. canicola,* produces the latter form. The relative incidence of the two infections in dogs is not known with certainty; some have suggested that as high as 50 per cent of canine leptospiroses are *icterohemorrhagiae* infections. *Lept. canicola* infection in dogs appears to be relatively common in the United States, and natural infection with other serotypes occurs also. Surveys[2] have shown that 13 to 32 per cent of dogs are seropositive, and 11 per cent of the positives are carriers of the infection. In addition to *Lept. canicola* and *Lept. icterohemorrhagiae,* which are found most often, dogs in this country are also infected with *Lept. grippotyphosa, Lept. pomona, Lept. autumnalis,* and *Lept. ballum.*

SWAMP FEVER

A leptospiral infection prevalent during the summer and early autumn months in Bavaria,[74, 84] Silesia, and in the Volga region[67, 83] is variously known as swamp fever, *Schlammfieber,* autumn fever, water fever, field fever, harvest fever, mud fever, and slime fever. It has also been reported from Holland. It is found among field workers following floods or in swampy districts particularly during the hay harvest. Clinically it closely resembles Weil's disease in a mild form—the case fatality is less than 1 per cent—but there is no jaundice except occasionally in the sclera. The causative microorganism, *Lept. grippotyphosa* (*Spirochaeta grippotyphosa*), is immunologically distinct from *Lept. icterohemorrhagiae.* The reservoir of infection is the field mouse, *Microtus arvalis;* the wood mouse, *Apodemus sylvaticus,* and the bank vole, *Evotomys glareolus,* are less often infected.

A very similar disease is found in the rice fields in Italy, but the etiology appears to be diverse in that a number of species of leptospira have been found, in many instances apparently identical with those found in the Far East. A similar situation holds true for *swineherd's disease*[59] (Bouchet-Gsell disease) found in Switzerland; pigs are infected with *Lept. pomona,* also found in Australia and in France,[58] and are the source of human infection. Infection of cattle and man with a leptospira closely related to but not identical with *Lept. grippotyphosa* has been reported from the Near East, and possibly the human infection may be contracted from cattle. Similar reservoirs of leptospirosis in wild animals, as

well as pigs, occur in the United States,[71, 72] and raccoons may have been the source of Fort Bragg fever which was found retrospectively to be an infection with *Lept. autumnalis.*

LEPTOSPIRAL INFECTIONS OF THE FAR EAST

A number of leptospiral infections occur in the Far East. They are similar to swamp fever in that they are febrile, jaundice is rare except in the sclera, and the case fatality rate is low. *Japanese 7-day fever,* or *nanukayami,* is prevalent in Japan in the autumn. The causative agent is *Lept. hebdomadis* and is carried by the field mouse, *Microtus montebelli.* A common sequel to the infection is opacification of the vitreous humor. *Hasami fever* is a similar disease also prevalent in Japan and caused by *Lept. autumnalis* (*Lept. akiyami* A). The reservoir of infection is the mouse *Apodemus speciosus,* and possibly other species of field mice and rats are infected.

Leptospirosis of heterogeneous etiology is not uncommon in Indonesia. The disease which occurs in Sumatra, which is known as *Rachmat infection,* is caused by *Lept. autumnalis* and is presumably identical with Hasami fever in Japan. Other infections are known by various names, including *Andaman A fever, Salinem infection,* and the like. The causative organism of Andaman fever appears to be *Lept. grippotyphosa.* The name *Lept. pyrogenes* has been given the causal agent of Salinem fever. The remainder of the leptospiroses appear to be largely infections with *Lept. bataviae* which is found in natural infections in rats, including *Rattus norvegicus* and *R. decumanus* (in Japan), and in field mice including *Apodemus sylvaticus, Micromys minutus,* and *M. soricinus.*

Mild fevers of leptospiral etiology are endemic in Queensland in Australia, and a number of immunologically distinct varieties of leptospira have been isolated. That designated *Lept. pomona* is the causative agent of the affection known as *Pomona fever,* and other species named *Lept. australis A* and *Lept. australis B* are responsible for febrile disease designated *Mossman fever, coastal fever,* and the like. Additional varieties have also been reported, but it is not as yet clear that they are distinct species.

Geographical separation of the leptospiroses is not sound for some of these microorganisms occur in widely separated regions. Thus *Lept. grippotyphosa* is found in Indonesia and *Lept. bataviae* and *Lept. pomona* have been reported as the etiological agents of swamp fever in Italy. *Lept. icterohemorrhagiae* and *Lept. canicola* occur in human beings and in dogs, and *Lept. pomona* has been found in naturally occurring infections of cattle in this country.

SAPROPHYTIC LEPTOSPIRA

Leptospira closely resembling *Lept. icterohemorrhagiae* have been isolated from water by a number of workers. These nonpathogenic forms have been termed *Lept. biflexa* in this country and in England, and *Spirochaeta pseudoicterogenes* by the German workers. Their relation to the pathogenic leptospira is uncertain.

RAT-BITE FEVER (SPIRILLUM MORSUS MURIS)

There are two distinct kinds of disease which may follow the bite of rats and are designated rat-bite fever. One, *Streptobacillus moniliformis* infection, is discussed elsewhere (Chap. 27). The other, known in Japan as *sodoku,* is caused by a spiral microorganism discovered by Futaki and his co-workers

in 1916. Following the bite of an infected rat, the original wound heals, but, after an incubation period of 10 to 22 days, becomes inflamed and painful. Fever, swelling of the lymph glands, skin eruptions, and other symptoms occur. The fever is of the relapsing type, with paroxysms at fairly regular intervals, usually about once a week, which continue to recur for 1 to 3 months or longer. The case fatality varies from 2 to 10 per cent.

The characteristic spiral microorganism has been found in the swollen local lesions of the skin and the enlarged lymph glands and also in two instances in the circulating blood. Guinea pigs and mice may be infected by the inoculation of blood or fluid expressed from the local lesions. The microorganism is found in about 3 per cent of the house rats in Japan. The rat is not the only vector of the infection, for cases of what is apparently the same disease have been traced to the bite of the cat, dog, pig, ferret, squirrel, and weasel.

The disease has long been known in Japan and has been reported from many localities all over the world. Brown and Nunemaker[17] have found a total of 125 cases reported in the United States from 1916 to the end of 1940. What proportion of the earlier reported cases were Streptobacillus infections is, of course, problematical. Of forty cases reported from 1931 to 1940, the spirillum was demonstrated by animal inoculation in seventeen; of these eleven resulted from rat bite and the remainder had a history of mouse bite, cat bite or scratch, contact with dogs, or trauma without known animal contact. The natural occurrence of spirochetes in laboratory mice, rats, and guinea pigs is a source of error to be guarded against in laboratory diagnosis by animal inoculation. There is little doubt that the affection is much more common than appears from the records, for the cases occur sporadically and their true nature has often gone unrecognized.[60] The infection has been produced in man by artificial inoculation for the treatment of paresis; according to Brown and Nunemaker there are 104 such cases in the English literature.

Figure 230. *Spirillum morsus muris* in blood of an inoculated mouse. (Van Sandt.)

Not a great deal is known of the immune response to the infection other than that a spirillicidal antibody is produced that is also responsible for immobilization of the spirilla in immune serum. Savoor and Lewthwaite[94] have observed that a positive Weil-Felix reaction of the OXK type is produced in experimental animals, but the antigen shared with these strains of Proteus is distinct from that which is responsible for the spirillicidal antibody.

The spiral microorganism of rat-bite fever has been variously classified as *Spirochaeta morsus muris, Borrelia muris, Spirillum minus,* etc. It is shorter —2 to 5 μ in length—than the recognized spirochetes, is relatively rigid, and possesses polar tufts of flagella which give it a rapid darting motion unlike the flexible undulating movements of the spirochetes. The clinical symptoms of the disease are typical of spirochetosis. Arsenicals have been used chemotherapeutically in the past, and the bacteria are sensitive to penicillin and the broad spectrum antibiotics; a combination of penicillin-tetracycline therapy was found highly effective in one instance.[60] Originally given the name Spirochaeta, this microorganism was found to be identical with *Spirillum minus* found by Carter in the blood of a rat in India. It is, perhaps, best given the independent generic name Spirillum and considered with the spirochetes.

REFERENCES

1. Addamiano, L., and Babudieri, B.: Research on spirochaetal strains isolated in Jordan. Bull. W. H. O., 1957, **17:**483–485.
2. Alexander, A. D., Gleiser, C. A., Malnati, P., and Yoder, H.: Observations of the prevalence of leptospirosis in canine populations of the United States. Amer. J. Hyg., 1957, **65:** 43–56.
3. Allen, R. H., and Tonks, D. B.: Pure phosphatides and the serodiagnosis of syphilis. Relation of chemical structure to reactivity. Bull. W. H. O., 1958, **19:**547–561.
4. Alston, J. M.: Recent developments in leptospirosis. Proc. Roy. Soc. Med., 1961, **54:**61–67.
5. Alston, J. M., and Broom, J. C.: Leptospirosis in Man and Animals. Williams and Wilkins, Baltimore. 1958.
6. Annear, D. I.: Observations on the preservation by drying of leptospirae and some other bacteria. Australian J. Exptl. Biol. Med. Sci., 1958, **36:**1–4.
7. Babudieri, B.: Schutzimpfung gegen Leptospirosen. Zent. f. Bakt., I Abt. Orig., 1957, **168:**280–283.
8. Babudieri, B.: Laboratory diagnosis of leptospirosis. Bull. W. H. O., 1961, **24:**45–58.
9. Baltazard, M., Bahmanyar, M., and Chamsa, M.: Sur l'usage du cobaye pour la différenciation des spirochètes récurrents. Individualité du group *B. crocidurae*. Bull. Soc. Path. Exot., 1954, **47:**864–877.
10. Beerman, H. et al.: Syphilis review of the recent literature 1959–1960. Arch. Intern. Med., 1961, **107:**121–140.
11. Bentzon, M. W., and Krag, P.: The international standard for human syphilitic serum. Bull. W. H. O., 1961, **24:**257–264.
12. Berger, U.: Die Treponemen der Mundhöle und ihre Bedeutung für die Pathogenese der oralen Fusospirochätosen. Beiträge z. Hyg. u. Epidemiol. Leipzig. 1958, No. 12, 139 pp.
13. Bohls, S. W., Irons, J. V., and DeShazo, T.: Cultivation of relapsing fever spirochetes in embryonic chick. Proc. Soc. Exptl. Biol. Med., 1940, **45:**375–377.
14. Bohls, S. W., and Schuhardt, V. T.: Relapsing fever in Texas and laboratory method of diagnosis. Texas State J. Med., 1933, **29:**199–203.
15. Bossak, H. N., Falcone, V. H., and Harris, A.: A year's experience with a nationwide TPI testing service. Pub. Health Repts., 1957, **72:** 317–320.
16. Broom, J. C.: Leptospirosis in tropical countries. A review. Trans. Roy. Soc. Trop. Med. Hyg., 1953, **47:**273–286.
17. Brown, T. McP., and Nunemaker, J. C.: Ratbite fever. A review of the American cases with re-evaluation of etiology; report of cases. Bull. Johns Hopkins Hosp., 1942, **70:** 201–327.
18. Brown, W. J.: A current look at venereal diseases. I. Current status of syphilis in the United States. Pub. Health Repts., 1960, **75:** 990–993.
19. Cannefax, G. R., and Garson, W.: The demonstration of a common antigen in Reiter's treponeme and virulent *Treponema pallidum*. J. Immunol., 1959, **82:**198–200.
20. Carpenter, C. M., Miller, J. N., and Boak, R. A.: A "triple-test plan" for the serologic diagnosis of syphilis—a modern-day approach. New England J. Med., 1960, **263:** 1016–1018.
21. Carpenter, C. M., Miller, J. N., Boak, R. A., and Heiskell, C. L.: The relationship of Brucella infection to biologic false positive tests for syphilis. Amer. J. Med. Sci., 1961, **241:**231–234.
22. Castellanos, A., and González Piñon, L.: El mal del pinto. Medicina, 1957, **37:**401–418.
23. Chargin, L., Sobel, N., and Rosenthal, T.: Treatment of early syphilis with penicillin, mapharsen and bismuth (combined therapy). Arch. Dermat. Syph., 1951, **63:**104–112.
24. Chorpenning, F. W., and Beers, D. B.: Biologic false positive reactions in serological tests for syphilis. Comparison of standard tests for syphilis with the treponemal immobilization test. J. Lab. Clin. Med., 1957, **49:**928–933.
25. Cockburn, T. A.: The origin of the treponematoses. Bull. W. H. O., 1961, **24:**221–228.
26. Cox, C. D.: Hemolysis of sheep erythrocytes sensitized with leptospiral extracts. Proc. Soc. Exptl. Biol. Med., 1955, **90:**610–615.
27. Cox, C. D., and Larson, A. D.: Colonial growth of leptospirae. J. Bact., 1957, **73:**587–589.
28. Curtis, A. C., et al.: Penicillin treatment of syphilis. J. Amer. Med. Assn., 1951, **145:** 1223–1226.
29. Curtis, A. C., and Schuster, D. S.: The laboratory diagnosis, biology and treatment of latent syphilis. J. Amer. Med. Assn., 1958, **167:**560–562.
30. D'Alessandro, G., and Del Carpio, C.: A lipopolysaccharide antigen of the Treponema. Nature, 1958, **181:**991–992.
31. Davis, G. E.: The spirochetes. Ann. Rev. Microbiol., 1948, **2:**305–334.
32. Davis, G. E., and Hoogstraal, H.: The relapsing fevers: A survey of the tick-borne spirochetes of Egypt. J. Egyptian Pub. Health Assn., 1954, **29:**139–143.
33. Deacon, W. E., and Freeman, E. M.: Fluorescent treponemal antibody studies. J. Invest. Dermat., 1960, **34:**249–253.
34. Deacon, W. E., Freeman, E. M., and Harris, A.: Fluorescent treponemal antibody test. Modification based on quantitation (FTA-200). Proc. Soc. Exptl. Biol. Med., 1960, **103:**827–829.
35. Degos, R., Vissian, L., and Basset, H.: Bilans cliniques et biologiques établis chez 1,340 syphilitiques (syphilis primo-secondaire) ayant suivi un traitement régulier d'au minimum quartre ans (arseno-bismuthique et bismuthique). Ann. Dermat. Syph., 1950, **10:**497–500.
36. Faine, S.: Iron as a growth requirement for pathogenic leptospira. J. Gen. Microbiol., 1959, **20:**246–251.
37. Fife, E. H., Jr., Bryan, B. M., Sanders, R. W., and Muschel, L. H.: Evaluation of the fluorescent treponemal antibody (FTA) test for syphilis. Comparison with *Treponema pallidum* immobilization (TPI) test. Amer. J. Clin. Path., 1961, **36:**105–113.
38. Fiumara, N. J.: Treponemal tests in diagnosis of syphilis and biologic false positive reactors. Pub. Health Repts., 1960, **75:**1011–1019.
39. Geigy, R., Wagner, O., and Aeschlimann, A.:

Transmission génitale de *Borrelia duttoni* chez *Ornithodorus moubata.* Acta Tropica, 1954, **11:**81–82.
40. Ghosh, R., and Ghosh, S.: A review of literature on pinta—a non-venereal treponematosis: With a note on the study of the disease in Bengal. J. Indian Med. Assn., 1959, **33:** 50–55.
41. Grin, E. I.: Endemic syphilis in Bosnia, W. H. O. Monograph Series No. 11. World Health Organization, Palais des Nations, Geneva. 1952.
42. Grin, E. I.: Endemic syphilis and yaws. Bull. W. H. O., 1956, **15:**959–973.
43. Guthe, T.: The treponematoses as a world problem. Brit. J. Venereal Dis., 1960, **36:** 67–77.
44. Hackett, C. J.: The transmission of yaws in nature. J. Trop. Med. Hyg., 1957, **60:**159–168.
45. Hackett, C. J.: An international nomenclature of yaws lesions. W. H. O. Monograph Series No. 36. World Health Organization, Palais des Nations, Geneva. 1957.
46. Hackett, C. J.: Some epidemiological aspects of yaws eradication. Bull. W. H. O., 1960, **23:**739–761.
47. Hackett, C. J., and Loewenthal, L. J. A.: Differential diagnosis of yaws. W. H. O. Monograph Series No. 45. World Health Organization, Palais des Nations, Geneva. 1960.
48. Harrison, L. W.: The origin of syphilis. Brit. J. Venereal Dis., 1959, **35:**1–7.
49. Hasselmann, C. M.: Comparative studies on the histopathology of syphilis, yaws and pinta. Brit. J. Venereal Dis., 1957, **33:**5–12.
50. Hemingway, M. W., Hemingway, R. W., and Arneson, V. K.: Relapsing fever. Northwest Med., 1940, **39:**362–364.
51. Heywood, C. P.: Interpretation of serum reactions in late syphilis. Brit. J. Venereal Dis., 1952, **28:**3–9.
52. Holcomb, R. C.: Pinta, a treponematosis: Review of the literature. U. S. Naval Med. Bull., 1942, **40:**517–552.
53. Hudson, E. H.: Non-venereal Syphilis. A Sociological and Medical Study of Bejel. Livingston, Edinburgh. 1958.
54. Hudson, E. H.: Historical approach to the terminology of syphilis. Arch. Dermat., 1961, **84:**545–562.
55. Jefferiss, F. J. G., Willcox, R. R., and McElligott, G. L. M.: Treatment of early syphilis with penicillin, neoarsphenamine and bismuth and with penicillin and bismuth alone. Lancet, 1951, **i:**83–85.
56. Kahn, R. L.: Universal serologic reaction with lipid antigen. Amer. J. Clin. Path., 1949, **19:** 347–368; 401–423.
57. Kirschner, L., and Graham, L.: Growth, purification and maintenance of leptospira on solid media. Brit. J. Exptl. Path., 1959, **40:** 57–69.
58. Kolochine-Erber, B., and Mailloux, M.: Les leptospiroses porcines en France. Seconde enquête et isolement de *Leptospira pomona.* Ann. Inst. Pasteur, 1960, **99:**359–375.
59. Kolochine-Erber, B., and Mailloux, M.: Les leptospiroses humaines à *L. pomona* et *L. mitis* Johnson 1942 (maladie des porchers). Ann. Inst. Pasteur, 1961, **100:**53–70.
60. Kowal, J.: Spirillum fever. Report of a case and review of the literature. New England J. Med., 1961, **264:**123–128.
61. Krag, P., and Bentzon, M. W.: The third international reference preparation of egg lecithin. Bull. W. H. O., 1961, **24:**265–269.
62. Leon y Blanco, F., and Oteiza, A.: The experimental transmission of pinta, mal del pinto, or carate to the rabbit. Science, 1945, **101:** 309–311.
63. MacArthur, W. P.: "The sibbens" or "the sivvens." Trans. Roy. Soc. Trop. Med. Hyg., 1953, **47:**437–438.
64. MacArthur, W. P.: Historical notes on some epidemic diseases associated with jaundice. Brit. Med. Bull., 1957, **13:**146–149.
65. Magnuson, H. J., et al.: Inoculation syphilis in human volunteers. Medicine, 1956, **35:**33–82.
66. Magnuson, H. J., and Portnoy, J.: The *Treponema pallidum* complement-fixation test. Amer. J. Pub. Health, 1956, **46:**190–194.
67. Markov, G. P., and Rybkina, L. G.: Cattle as a source of icterohaemorrhagic leptospires. J. Microbiol. Epidemiol. Immunobiol., 1957, **28:**393–396. (Translated from the Russian.)
68. Marquez, F., Rein, C. R., and Arias, O.: Mal del pinto in Mexico. Bull. W. H. O., 1955, **13:**299–322.
69. Martini, E.: Zür älteren Geschichte der Recurrens im europäischen Raum. Ergebn. Hyg. Bakt. Immunitätsf., 1955, **29:**213–247.
70. Masterson, G.: A case of third generation syphilis. Brit. J. Venereal Dis., 1956, **32:**171–174.
71. McKeever, S., et al.: Incidence of leptospirosis in wild animals from southwestern Georgia, with a report of new hosts for six serotypes of leptospires. Amer. J. Trop. Med. Hyg., 1958, **7:**646–655.
72. McKeever, S., Gorman, G. W., Galton, M. M., and Hall, A. D.: The raccoon, *Procyon lotor,* a natural host of *Leptospira autumnalis.* Amer. J. Hyg., 1958, **68:**13–14.
73. Merriweather, A. M.: Endemic syphilis—"dichuwa"—in the Bechuanaland Protectorate. Central African J. Med., 1959, **5:**181–185.
74. Mochmann, H., and Hentschel, G.: Erfahrungsbericht über eine Schlamm-Feldfieber-Epidemie des Jahres 1955 in Mitteldeutschland und ihre Beziehungen zu Klima und Witterung. Zent. f. Bakt., I Abt. Orig., 1958, **170:**583–596.
75. Montgomery, C. H., Suhrland, S., and Knox, J. M.: Observations concerning fluorescent treponemal antibody test for syphilis. J. Invest. Dermat., 1960, **35:**95–101.
76. Mooser, H.: Die Rückfallfieber. Ergebn. Mikrobiol. Immunitätsf., 1958, **31:**184–228.
77. Moschella, S. L.: The "unitarian concept" of the treponemal diseases. U. S. Armed Forces Med. J., 1956, **7:**1101–1108.
78. Nelson, R. A., Jr., and Diesendruck, J. A.: Studies on treponemal immobilizing antibodies in syphilis. I. Techniques of measurement and factors influencing immobilization. J. Immunol., 1951, **66:**667–685.
79. Nelson, R. A., Jr., and Mayer, M. M.: Immobilization of *Treponema pallidum* in vitro by antibody produced in syphilitic infection. J. Exptl. Med., 1949, **89:**369–393.
80. Pangborn, M. C., et al.: Cardiolipin antigens. Preparation and chemical and serological control. W. H. O. Monograph Series No. 6. World Health Organization, Palais des Nations, Geneva. 1955.
81. Pike, R. M., and Schulze, M. L.: Serologic variants of leptospira types resulting from growth

in immune serum. J. Immunol., 1958, **81:**172–175.
82. Pollock, J. S. McK.: Sibbens or sivvens—the Scottish yaws. Trans. Roy. Soc. Trop. Med. Hyg., 1953, **47:**431–436.
83. Popova, E. M., and Amosenkova, N. I.: Reservoirs of leptospiral infection in the northwest regions of the U.S.S.R. Results of an investigation on leptospiral infections in murine rodents. J. Microbiol. Epidemiol. Immunobiol., 1957, **28:**44–49. (Translated from the Russian.)
84. Popp, L.: Die Epidemiologie des Feldfiebers im niedersächsischen Gebirgsvorland. Arch. f. Hyg. u. Bakt., 1960, **144:**345–374.
85. Portnoy, J., and Magnuson, H. J.: Immunologic studies with fractions of virulent *Treponema pallidum*. I. Preparation of an antigen by desoxycholate extraction and its use in complement fixation. J. Immunol., 1955, **75:**348–355.
86. Puccinelli, V.: Plurality of antibodies in syphilitic serum and clinical practice. Brit. J. Venereal Dis., 1952, **28:**184–188.
87. Reiss-Gutfreund, R. J.: Culture de *Borrelia recurrentis* (souches éthiopiennes) en oeuf féconde de poule. Ann. Inst. Pasteur, 1960, **98:**131–136.
88. Report: Diagnosis and typing in leptospirosis. Report of a Study Group. Amsterdam. W. H. O. Technical Report Series No. 113. World Health Organization, Palais des Nations, Geneva. 1955.
89. Rice, F. A. H.: Composition of cardiolipin. Science, 1958, **127:**339–340.
90. Rose, N. R., and Morton, H. E.: The morphologic variation of treponema. Amer. J. Syph., 1952, **36:**17–37.
91. Rothstein, N.: Studies of the immunochemistry of leptospires. II. Heterogenetic relationship between leptospires and other microorganisms. J. Immunol., 1957, **79:**276–280.
92. Ruge, H. G. S.: Role of Treponema Reiter in modern serology of syphilis. Brit. J. Venereal Dis., 1956, **32:**242–245.
93. Sanders, R. W., Piper, D. R., Hook, W. A., and Muschel, L. H.: Nonspecific immobilization in the *Treponema pallidum* immobilization (TPI) test. Amer. J. Clin. Path., 1960, **33:**135–137.
94. Savoor, S. R., and Lewthwaite, R.: The Weil-Felix reaction in experimental rat bite fever. Brit. J. Exptl. Path., 1941, **22:**274–292.
95. Schuhardt, V. T., and Wilkerson, M.: Relapse phenomena in rats infected with single spirochetes (*Borrelia recurrentis* var. *turicatae*). J. Bact., 1951, **62:**215–219.
96. Shirvinskaia, A. I.: Study of type-specific immunity in experimental leptospirosis. J. Microbiol. Epidemiol. Immunobiol., 1959, **30:**39–43. (Translated from the Russian).
97. Simpson, C. F., and White, F. H.: Electron microscope studies and staining reactions of leptospires. J. Infect. Dis., 1961, **109:**243–250.
98. Simpson, W. G., Harris, A., Garson, W., and Bunch, W. L., Jr.: The Reiter protein complement fixation test as a diagnostic aid in syphilis. Arch. Dermat., 1960, **81:**904–907.
99. Sosa-Martínez, J., and Peralta, S.: An epidemiologic study of pinta in Mexico. Amer. J. Trop. Med. Hyg., 1961, **10:**556–565.
100. Sparrow, H.: Les spirochètes des fièvres récurrentes et le pou (de l'origine des épidémies de récurrente à poux). Ann. Inst. Pasteur de Tunis, 1955, **32:**25–49.
101. Stein, G. J.: The serological diagnosis of relapsing fever. J. Exptl. Med., 1944, **79:**115–128.
102. Swain, R. H. A.: Electron microscopic studies of the morphology of pathogenic spirochaetes. J. Path. Bact., 1955, **69:**117–128.
103. Swain, R. H. A.: The electron-microscopical anatomy of *Leptospira canicola*. J. Path. Bact., 1957, **73:**155–158.
104. Symposium: A Symposium on Relapsing Fever in the Americas. American Association for the Advancement of Science, Publication No. 18. 1942.
105. Symposium: Symposium on yaws. Bull. W. H. O., 1953, **8:**1–418.
106. Symposium: Serology of syphilis. Bull. W. H. O., 1956, **14:**187–351.
107. Symposium: Animal disease and human health. Part III. Leptospirosis: A contemporary problem. Ann. N. Y. Acad. Sci., 1958, **70:**391–444.
108. van Thiel, P. H.: Are spirochaetes bacteria or protozoa? Antonie van Leeuwenhoek. J. Microbiol. Serol., 1959, **25:**161–168.
109. Thompson, F. A., Jr., and Magnuson, H. J.: Studies on increasing the sensitivity of the treponemal immobilization test for syphilis. Amer. J. Syph., 1951, **35:**21–34.
110. Thomson, I. G.: The pathogenesis of tropical ulcer amongst the Hausas of Northern Nigeria. Trans. Roy. Soc. Trop. Med. Hyg., 1956, **50:**485–495.
111. Turner, L. H.: Notes on the treponematoses with an illustrated account of yaws. Bull. Inst. Med. Res. Fed. Malaya, 1959, No. 9. 278 pp.
112. Turner, T. B., and Hollander, D. H.: Biology of the treponematoses. W. H. O. Monograph Series No. 35. World Health Organization, Palais des Nations, Geneva. 1957.
113. Villela, E.: Pinto y pinta. Medicina, 1952, **32:**34–38.
114. Weber, M. M.: Factors influencing the in vitro survival of *Treponema pallidum*. Amer. J. Hyg., 1960, **71:**401–417.
115. Widelock, D., et al.: The USR test as a screening test in a public health laboratory. Pub. Health Repts., 1961, **76:**447–452.
116. Willcox, R. R.: Evolutionary cycle of the treponematoses. Brit. J. Venereal Dis., 1960, **36:**78–91.
117. Wolff, J. W.: The Laboratory Diagnosis of Leptospirosis. Thomas, Springfield, Illinois. 1954.
118. Wolff, J. W., and Broom, J. C.: The genus Leptospira Noguchi, 1917. Problems of classification and a suggested system based on antigenic analysis. Documenta Med. Geograph. Trop., 1954, **6:**78–95.

Chapter 34

MEDICAL PARASITOLOGY

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THE DIVISION OF microbiology termed medical parasitology encompasses the animal agents of human disease. It may seem incongruous to include in "microbiology" organisms such as the larger helminths whose dimensions are measured in meters or liters, as is the case with the cestodes Diphyllobothrium and Echinococcus. There is, however, an entire spectrum of dimensions of parasitic animals, including the intracellular protozoans, only a few microns in diameter. Within the large group of phylogenetically dissimilar animals parasitizing man, parallel evolution has resulted in numerous instances of superficial similarity of form and host-parasite relationship. Physiological interactions with and dependencies on the host occur which we more commonly attribute to bacteria, viruses, and rickettsiae.[7, 135]

These similarities include such obvious disease production as anemias, dysenteries, encephalitis, and pneumonitis, as well as more obscure effects as hypersensitive and allergic responses. However, in contrast to other parasites, phylogenetically related parasitic animals are typified by variety and contrast rather than unifying principle in their disease production, physiology, morphology, life cycles, and other characteristics.

Knowledge of the large parasitic roundworms such as Ascaris and Dracunculus and the larger tapeworms and flukes is evident in the earliest writings, and the elucidation of a number of complex parasite life histories and appropriate control measures preceded much of our detailed knowledge of other microbial disease.[62, 117]

Descriptive parasitology has been a productive division of zoological science, resulting in a tremendous body of knowledge on the complex life cycles and morphology of these forms. This supplied the knowledge of the minute morphologic detail needed for parasite identification for, in contrast to the bacteria, diagnostic, metabolic or culture characteristics were largely unobtainable by usual procedures.[86, 120] Investigations of parasite physiology have been difficult because of inability to cultivate most forms free of their hosts. Some demand several hosts, each physiologically necessary to the cyclic development of the parasite, and difficulties of culture might be anticipated. Others of such apparent morphologic and cyclic simplicity as the intestinal amebae have also resisted intensive research directed at their cultivation on defined media in the absence of other living cells.

For these reasons, techniques of immunization, effective chemotherapy, and practical control measures have lagged behind those developed to combat other disease agents. Consequently, the current world incidence of infection and disease produced by parasitic animals

remains high and in some areas is increasing. Even in the case of malaria, which is being attacked on a global scale, annually 1.2 billion individuals live in areas where they are exposed to infection, 200 million cases are reported, and at a conservative estimate 1.5 million deaths result.[105, 110] Among the helminths the most important example is the schistosome complex which currently parasitizes approximately 4.5 per cent of the world's population and is increasing its distribution and prevalence in much of its range.[89, 106] The roundworms parasitizing man are so successful that the number of infections exceed the world population. Although many of these singly are relatively benign or only slightly debilitating to an otherwise healthy host, multispecies infections are the rule in many areas of the world where their effects are further enhanced by subsistence nutritional levels of the host.

Parasitic animals are of such importance to tropical medicine that this has resulted in the popular misconception that parasitic disease of man is exclusively a problem of the tropics.[84, 119] Natural transmission of Ascaris and Trichinella extends into arctic areas, and the classic endemic areas of hydatid (Echinococcus) disease are sheep raising areas including Iceland.[24, 40, 41, 69] Until the relatively recent past the midwestern United States and much of Europe were malarious areas.[57] Although more common in tropical areas, autochthonous amebic dysentery persists in causing individual and occasionally epidemic problems in northern urban communities,[88, 98] while Enterobius and Hymenolepis are dependent on the urbanization of man rather than on climate for survival. In addition, because of increased travel and protracted incubation periods of many parasitic diseases, numerous isolated instances of infection with exotic species are diagnosed in all major northern urban areas.

Disease-producing parasites of man are found among the unicellular animals (protozoa), the worms (platyhelminthes, nemathelminthes, and acanthocephala) and the arthropods. The latter includes many species of insects and arachnids of importance as parasites, disease vectors, intermediate hosts, and forms damaging by virtue of their toxic chemical, traumatic, or sensitizing properties. Only those of importance as intermediate hosts of parasites will be included in this chapter. It is not possible here to present material on the arthropods as disease agents and reference is made to various texts on the subject for full coverage.[59, 65] Over 150 protozoan and helminth parasites have been reported from man with approximately one third of these being common associates. Representatives have been selected for inclusion in this chapter to serve as illustrative examples or for their intrinsic importance in disease production. References for further study and more detailed coverage include standard clinical texts and selected texts and papers cited.[24, 40, 41, 69]

THE PROTOZOA

The protozoa are usually thought of as single-celled animals, analogous to the individual cells composing the higher animals. Some authorities, however, prefer to regard them as acellular organisms, not functionally subdivided into cells. Both views have value, for the protozoa evince properties both of single cells and of complete organisms.

Many thousand species of protozoa have been described, though less than thirty-five well-defined species are known to parasitize man. They constitute an exceedingly heterogeneous group of organisms, varying in size from that of the larger bacteria to several millimeters in diameter, in complexity of structure from a simple, formless cell like *Ent-*

amoeba histolytica to organisms of far greater intricacy than many metazoa, and in life cycle from the binary fission of *Trichomonas vaginalis* to the alternation of hosts and of asexual and sexual reproduction exhibited by the malarial parasites.[76, 78, 85]

The systematics of the protozoa is complex[79] and no single scheme of classification is uniformly acceptable. Division of the phylum Protozoa into four subphyla plus a small group with uncertain affinities provides a satisfactory simplified method of encompassing the species parasitizing man.

Subphylum Sarcodina (amebae), characterized by the ability to produce from the cell transient finger-like protoplasmic processes (pseudopodia) for the engulfment of food and for locomotion. Many free-living species as well as parasitic forms exist. The parasites characteristically are simple in appearance without morphologically complex organelles and multiply without any known sexual stages. All have a motile trophozoite stage during which multiplication is by binary fission. Many species also form a cyst in which stage two or more nuclear divisions may take place preceding the multiplication effected when the cyst infects the host. Amebae of man are primarily found in the digestive tract.

Subphylum Ciliophora (ciliates), having numerous, short, bristle-like, cytoplasmic locomotor organelles termed cilia. Most have two types of nuclei (macronucleus and micronucleus) with multiplication by binary fission and with conjugation in some forms. There are many free-living, commensal and symbiotic species with but a single form, *Balantidium coli,* occurring as a bona fide parasite of man.

Subphylum Mastigophora (the flagellates), characteristically having throughout or at some point in their life cycle relatively long, filamentous protoplasmic processes used in locomotion. These flagella may be multiple or single. Those intracellular parasitic stages lacking flagella may be recognized by a parabasal body, a rod-like structure associated with the flagellar origin. Division is by binary fission. Evidence for a sexual cycle in the hemoflagellates has been presented but is not wholly acceptable. Numerous free-living, parasitic, and commensal species exist. Flagellates parasitizing man are found in the intestinal and genital tracts, free in the circulation, and as intracellular parasites primarily of the lymphoid-macrophage system.

Subphylum Sporozoa, an artificial polyphyletic grouping of parasites characterized by the lack of well-defined organelles of locomotion and with an alternation of sexual and asexual reproduction. All members of this group are parasitic and many produce structures of varied morphology which have been designated as spores and which contain one to many infective individuals termed sporozoites. The malaria parasites (Plasmodia) and the intestinal coccidia of man belong to this group.

A separate group superficially similar to the sporozoans, of uncertain affinities, has been characterized as a class, Toxoplasmasida, having no spores and with asexual reproduction. This group would include Toxoplasma and Sarcocystis of man as well as a variety of parasites of other vertebrates.[78, 79]

To supplement this skeleton classification and the material to follow, a number of basic texts and taxonomic studies are available.[76, 85]

THE INTESTINAL AMEBAE (SARCODINA)

Entamoeba Histolytica. Lewis in 1870 and Cunningham in 1871 first reported amebae, probably the nonpathogenic *Entamoeba coli,* in human feces. Lösch in 1875 described what were apparently *E. histolytica* in the stools and intestinal ulcers in a fatal case of dysentery. He found similar ulcers containing the amebae in an artificially infected dog.

Characteristics and life cycle. The active ameba as seen in the intestinal ulcers of dysentery cases is a granular colorless or pale greenish mass of cytoplasm, 15 to 50 (usually 20 to 30) μ in diameter. It has no definite shape. Locomotion is accomplished by the sudden extrusion of clear projections of cytoplasm, the *pseudopodia,* the remainder of the cell body following these pseudopodia in a flowing motion. The granular cytoplasm often contains red blood cells or debris of tissue cells in various stages of digestion. These are the food engulfed by the ameba. The nucleus may be visible as a delicate ring of granules. Reproduction in this stage is by binary fission, the nucleus undergoing a type of mitosis and the cytoplasm then dividing to produce two daughter amebae like the original. In organisms fixed and stained with hematoxylin the nuclear structure is characteristic, consisting of a

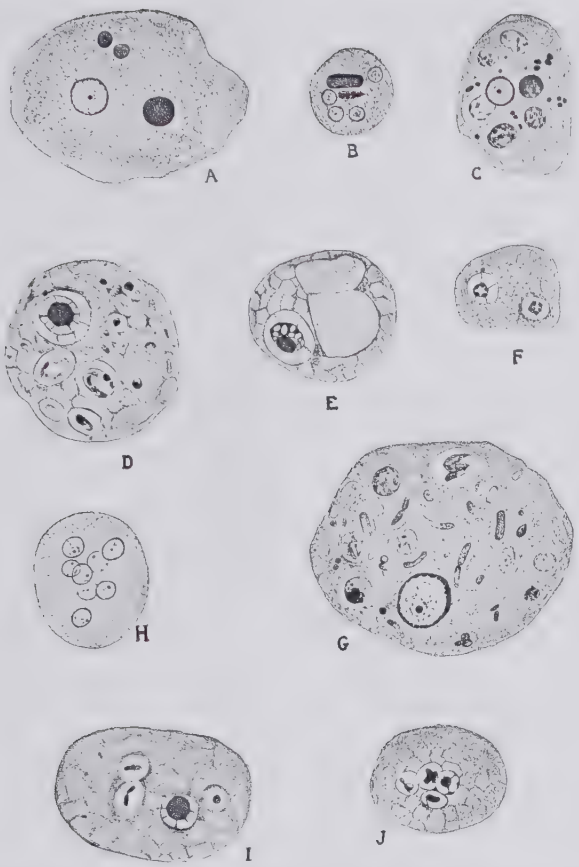


Figure 231. The amebae living in man. *A*, active ameboid form of *Entamoeba histolytica* containing three red blood cells. *B*, mature quadrinucleate cyst of the same containing two chromatoids. *E. hartmanni* is similar but has smaller nuclei. *C*, active ameboid form of *Entamoeba gingivalis*. *D*, active ameboid form of *Iodamoeba williamsi* containing many intestinal bacteria. *E*, cyst of same showing large double vacuole which in life was filled with glycogen. *F*, ameboid form of *Dientamoeba fragilis* containing two nuclei. *G*, large active ameboid form of *Entamoeba coli* containing intestinal bacteria and debris. *H*, mature octonucleate cyst of same. *I*, ameboid form of *Endolimax nana*. *J*, mature quadrinucleate cyst of same. *A*, *B*, *C*, *G*, and *H* × about 1300 (Dobell). *D*, *E*, *F*, and *J* × 3000 (Taliaferro and Becker). *I* × 3000. (Taliaferro in Hegner and Taliaferro's *Human Protozoology*, courtesy of The Macmillan Company.)

thin peripheral layer of fine black granules and a central small black dot, the *karyosome* (Fig. 231). The entire nucleus is generally 4 to 5 μ in diameter.

Infected persons with diarrhea or dysentery pass active ameboid parasites in their stools. In the intestinal lumen of a *carrier*, however, the ameba loses its ingested food particles, shrinks to a diameter of 10 to 20 μ (rarely less), rounds up and becomes essentially nonmotile, sending out only an occasional pseudopodium. This is the *precystic stage*, which soon secretes about itself a clear wall, becoming the partially resistant *cyst*. In its passage down the intestine the cyst continues to develop, acquiring a vacuole of glycogen and one or more ovoid rods of black-staining material, the *chromatoid bodies*. The nucleus divides into two and then four, all resembling the nucleus of the active ameboid stage though considerably smaller. In the mature cyst the glycogen vacuole soon disappears, and the chromatoid bodies persist at most for a few days.

The cyst is the infective stage, since most or all of the active ameboid stages are destroyed by gastric juice. As studied in culture excystment consists of

the emergence of a four-nucleate organism which by a complicated division process produces eight small amebae. These are the stages which initiate a new infection.

Boeck and Drbohlav in 1925 cultivated *E. histolytica* in Locke's solution and inactivated serum tubed over egg slants. Various modifications have been introduced, the most important being the addition of powdered rice starch, which the amebae ingest avidly. Cleveland and Collier used liver infusion agar slants overlaid with serum-saline. Balamuth devised a valuable monophasic medium consisting of buffered aqueous egg yolk infusion, with or without liver extract.

These mediums do not permit pure culture, for the amebae are dependent on living bacteria for growth. The protozoan *Trypanosoma cruzi* has also been utilized as an associate for culture of the amebae. Shaffer and Frye obtained amebic growth in the presence of antibiotic-inhibited bacteria. Cultivation of a strain of *E. histolytica* for six months with 50 transfers in a cell-free system containing chick embryo extract has been reported. This is the first extensive truly axenic cultivation of this species, although not yet applicable to all strains.[34] The related tissue-invading, pathogenic parasite of snakes, *E. invadens,* has been cultivated successfully in a cell-free liver extract medium. *E. histolytica* is a strict anaerobe and requires a pH between 6.5 and 7.0 approximately.[87]

Amebiasis and amebic dysentery. The amebae normally inhabit the large intestine. While some workers believe that they proliferate only in the tissues, the evidence indicates that they may also multiply in the intestinal lumen, living, as in culture, on a diet of microorganisms.[60]

In the wall of the colon the parasites are found in necrotic, usually noninflammatory lesions varying from minute erosive patches to more or less extensive, undermined ulcers of the submucosa. The abundance and severity of the lesions determine the clinical picture. The great majority of infected individuals are carriers exhibiting no symptoms. Clinical cases range from moderate diarrhea to acute dysentery with passage of blood and mucus, extreme weakness, and, not infrequently, death. A varying proportion of individuals with amebic dysentery show necrotic abscesses of the liver, or may show a generalized hepatitis of amebic etiology. Initially the liver abscesses are bacteria-free but may become contaminated secondarily.[77, 82] These also occur occasionally in individuals who give no history of intestinal symptoms of infection. Lung abscesses, usually produced by extension from the liver through the diaphragm, occur in a small number of individuals, and abscesses have been reported in practically every organ of the body.

The entire question of host-parasite relationships in amebiasis including disease production is complex and controversial.[60, 90] The lack of agreement even in the morphological bases for diagnosis of the species causing amebic disease has also complicated the picture.[16, 22, 108] Strains of the species vary in their morphology and in their pathogenicity for man.[61, 95, 96] Some rapidly lose pathogenicity in culture, and human pathogens may or may not be pathogenic for laboratory animals. Strains of human origin, pathogenic for animals, have been shown to be completely innocuous in human volunteers.[9] Some investigators have presented evidence that the pathogenicity of amebae is directly related to the pathogenicity of the bacterial associates, while others refute this. The dependence of most strains of amebae in culture on bacterial associates and the inability of amebae to produce extensive lesions in bacteria-free guinea pigs lend weight to the concept of the need for synergistic bacterial associates in amebiasis.[81, 101] However, as mentioned earlier, sterile amebic abscesses are found. The suggestions that individual variation in resistance or that nutritional factors are of primary importance in the severity of the disease have supporters but are also disputed.[36, 45] Attempts have also been

made to associate invasiveness of strains to the presence or lack of protease or mucopolysaccharidase activity with equivocal results.[70, 96]

Immunity. There is no direct evidence of acquired immunity to infection with *E. histolytica.* Natives of hyperendemic regions show acute dysentery less frequently than aliens, possibly as a result of acquired immunity. Serum antibody response to the infection is evidenced by the complement-fixation test, which is positive in over 80 per cent of infected individuals. Sera from infected individuals will inhibit to varying degrees the ability of amebae in culture to ingest erythrocytes or can be shown to immobilize their locomotor activities.[118] Antibodies from infected individuals when labeled by fluorescein have considerable specificity for the infecting species and a degree of strain specificity.[53, 54] Immune precipitates in a gel diffusion system on a micro scale also show species specificity. As with similar systems for parasitic infections it is not possible at present to correlate these diagnostic serologic phenomena with functional immunity.

Diagnosis. Diagnosis of infection with *E. histolytica* depends upon the finding of characteristic organisms in the stools and their morphological differentiation from the nonpathogenic amebae occurring in human feces. Cultivation has been utilized, but in cultures *E. histolytica* resembles the nonpathogenic *E. coli* so closely that differential diagnosis is often very difficult. Direct smears of fresh loose stools in warm saline may reveal the active ameboid stages. They may also be found in material taken directly from lesions in the lower bowel with the aid of the proctoscope. The M.I.F. stain of Sapero, Lawless, and Strome is valuable for temporary preparations, although permanent smears stained with iron hematoxylin give the best differentiation. In well-formed stools cysts may be expected, and these are most easily identified in iodine solutions, as D'Antoni's iodine. The formalin-ether concentration method of Ritchie (for cysts) and the M.I.F. concentration method of Blagg et al. (for both cysts and trophozoites) are excellent for diagnosis of the light infections which may be missed by direct examination. The P.V.A. preservation technique of Brooke et al. permits shipment of material to a central laboratory for examination.[16] *E. histolytica* appears irregularly in the stools and, whatever the technique, repeated examinations are often necessary to establish a diagnosis. It should be re-emphasized that recovery of the organisms is only the first step in diagnosis of *E. histolytica* infection. The final step is the identification of the parasites, a task requiring abundant experience. Some concept of the variation between individuals can be gained by examining photomicrographs.[120] However, considerable experience is needed to exclude the commensals and smaller related forms such as *E. hartmanni.* The varied symptomatology and severe nature and importance of amebic disease necessitates the development of a more objective diagnostic method than presently exists. Axenic cultivation procedures and immuno-cytochemical methods being studied may produce this in the future.

Chemotherapy. Emetine has a favorable effect on the symptoms but rarely eliminates the infection from the intestine. Several iodine-containing drugs, notably chiniofon and Diodoquin, and arsenicals, especially carbarsone and Milibis, are effective in eradicating the amebae.

Several antibiotics are useful in treatment. Chlortetracycline is moderately efficient, and oxytetracycline cures about 90 per cent of cases. Chloroquine and Camoquin, while useless in intestinal amebiasis, are highly curative in extraintestinal infection.

Epidemiology and control. The active ameboid stages of *E. histolytica* die quickly after exit from the body, for they are very susceptible to drying and to changes in temperature and salt concentration. Since they are destroyed by gastric juice they are not usually infective when swallowed. The amebic dysentery patient is therefore practically

harmless as a source of infection, since only the ameboid stages occur in his stools. The cysts passed by carriers, while not at all comparable to bacterial spores in resistance, show considerably less susceptibility to conditions outside the body than do the ameboid stages. Studies utilizing cysts from culture tested for viability by cultivation show survival of several months in water at 0° C., 3 days at 30° C., 30 minutes at 45° C. and 5 minutes at 50° C. Cysts of *E. histolytica* are somewhat more resistant to chlorine than are enteric bacteria. It is believed that ordinary residual chlorine concentrations are unable to destroy amebic cysts, but that hyperchlorination is effective. In this connection it should be borne in mind that in the Chicago amebic dysentery outbreak of 1933 there was abundant circumstantial evidence that the infection was spread by water containing sufficient residual chlorine to kill intestinal bacteria.[88]

In general, the spread of *E. histolytica* resembles that of intestinal bacterial infections, utilizing any means by which fecal contamination reaches the human mouth. Most important are drinking water, food handlers, and houseflies. Swimming pools, though not definitely incriminated, are a potential source of infection. Viable cysts have been found in the droppings of houseflies 1 to 2 days after exposure, and flies have been held responsible for at least one important outbreak. The 1933 epidemic in Chicago referred to above was traced to local sewage contamination of drinking water in two hotels. This epidemic, in which 1409 cases were discovered and there were 98 deaths, was the first major outbreak in a city of the temperate zone. It directed medical attention to a problem which had been considered important only in the tropics. As recently as 1955 a similar outbreak occurred in South Bend, Indiana, with contaminated drinking water as the source, re-emphasizing the fallacy in considering amebiasis an exotic infection. [98]

The distribution of *E. histolytica* is world-wide, but temperate regions have usually a low incidence of infection. Surveys indicate a general infection rate in the United States of 4 to 10 per cent, though in some southern localities the incidence has approached 40 per cent. These figures include *E. hartmanni* (see below) and are probably about twice the incidence of *E. histolytica* alone. In the tropics the carrier rate is generally very high, often exceeding 50 per cent. Human carriers are the only important source of infection, although *E. histolytica* occurs naturally in lower animals, particularly rats, dogs, and monkeys. The most widely used experimental animals are dogs, cats, monkeys, rats, guinea pigs, rabbits, and hamsters.[61]

Control of the spread of *E. histolytica* is not basically different from that of other human enteric infections. The high incidence of carriers not known to have had clinical dysentery complicates the problem, but it is ultimately a matter of prevention of access of human feces to the mouths of susceptibles.

Other Species of Amebae Parasitic in Man. Six other species of amebae live in the human intestine. With the exception of *Dientamoeba fragilis* these are nonpathogenic and are of medical interest only because they must be differentiated from *E. histolytica.*

E. hartmanni, long considered a small race of *E. histolytica,* has recently been shown to be a distinct species. It differs from *E. histolytica* in size, the cysts being always less than 10 μ in diameter, and in size of the nuclei, which are slightly more than half the diameter of those in *E. histolytica.* Usually it is not distinguished from *E. histolytica* in diagnostic work, but in a few surveys it has been found to make up about half the infections of the histolytica type. It is nonpathogenic and can be cultured only with difficulty.

Entamoeba coli, a common species, differs from *E. histolytica* in several characters. The stained nucleus shows thicker peripheral chromatin blocks and a larger and usually noncentral karyosome. The ameboid stage is sluggishly and usually nonprogressively motile with blunt, slowly extruded pseudo-

podia. It ingests bacteria and other particles but rarely red blood cells. The spherical cysts average somewhat larger, 10 to 33 μ in diameter, contain eight nuclei in the mature stage and may show chromatoid bodies with pointed or "splintered" ends.

E. polecki, a parasite of hogs and monkeys similar to *E. coli* but producing uninucleate cysts, has been found very rarely in man.

Endolimax nana is smaller, 6 to 15 μ in diameter in the ameboid stage. The stained nucleus shows no peripheral chromatin but a very large, nearly central karyosome. Movement is sluggishly progressive, and bacteria are ingested. The spherical or ovoid cyst is 5 to 14 μ in diameter, containing one to four minute nuclei and sometimes small spherical or rod-like chromatoid bodies.

Iodamoeba bütschlii measures 8 to 20 μ in diameter in the ameboid stage. The stained nucleus shows a large central karyosome surrounded by a layer of granules. Movement and inclusions are like those of *E. coli.* The cyst is irregular in shape, 5 to 20 μ in diameter, and contains one or rarely two nuclei. Minute granules may be seen, but the most striking feature of the cyst is a large glycogen mass, staining dark brown with iodine.

Dientamoeba fragilis, seldom reported in the general population though sometimes common in institutions, is a very small form, 5 to 12 μ in diameter. It usually shows two nuclei, each containing a large multiple karyosome. It moves actively, ingesting bacteria. It has been associated with acute dysentery and it has been suggested that it may produce low grade constant irritation with fibrotic changes producing an appendicitis.[123] It is considered by some protozoologists to be an aberrant flagellate rather than a true ameba. No cyst stage is known and the binucleate trophozoite, as its name suggests, is readily destroyed. One investigation suggests that *D. fragilis* is transmitted via the egg of the pinworm of man, *Enterobius vermicularis.*[21] Precedent for this is found in the cycle of *Histomonas meleagridis,* an ameba-like, pathogenic flagellate of the turkey which is transmitted through an Ascarid egg.

E. gingivalis, probably the first parasitic ameba seen, was reported by Gros in 1849 from the tartar between the teeth. It has no known cyst stage and is apparently transmitted in the ameboid stage by contact. Formerly suspected of an etiological role in pyorrhea, it is now considered harmless.

CILIOPHORA

Balantidium coli is the largest protozoan parasite found in the intestine of man, reaching a size of 150 $\times$ 120 μ. The organism may cause an acute bloody dysentery similar to amebiasis, commonly penetrating into the muscularis mucosa and occasionally perforating the intestine.[5] Carrier infections without symptoms occur in man, and the world incidence is estimated at less than 0.7 per cent. Swine are almost universally infected and human infection usually derives from food or water contaminated with the resistant cysts

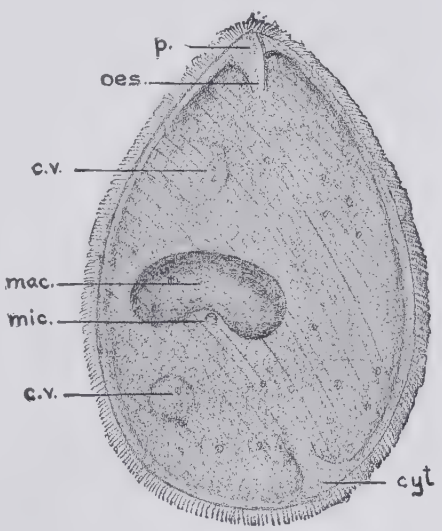


Figure 232. *Balantidium coli* from man: *c.v.,* contractile vacuole; *cyt.,* cytopyge; *mac.,* macronucleus; *mic,* micronucleus; *oes.,* esophagus; *p.,* peristome. (Hegner in Hegner and Taliaferro's *Human Protozoology,* courtesy of The Macmillan Company.)

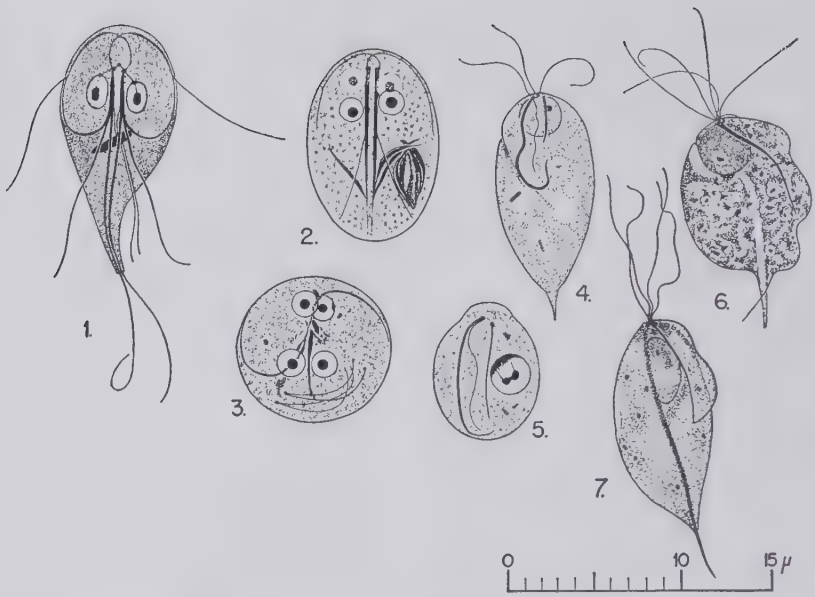


Figure 233. Flagellates of the human intestine and genital tracts, hematoxylin stain. *1, Giardia lamblia* trophozoite. *2, 3, G. lamblia* cysts. *4, Chilomastix mesnili,* trophozoite. *5, C. mesnili,* cyst. *6, Trichomonas hominis. 7, T. vaginalis* (Mackie, Hunter, and Worth, *A Manual of Tropical Medicine.* Ed. 2.)

from this source. Moderate to severe pathology is produced in other primates and infections naturally occur in the rhesus monkey, chimpanzee, dog, and Norway rat. *Balantidium* may be cultivated on a variety of media. Carbarsone is an effective chemotherapeutic agent, although Diodoquin and the tetracyclines have also been used successfully in treatment.

INTESTINAL FLAGELLATES (MASTIGOPHORA)

The mastigophora of the digestive tract and genital organs, illustrated in the accompanying figure, are typically lumen parasites. The common cosmopolitan species of enteric flagellates, *Chilomastix mesnili, Trichomonas hominis,* and *Giardia lamblia,* are host specific although a number of less common and less specific coprophagic forms are found in man. None are generally considered as important pathogens. However, Giardia is commonly associated with erosion of the epithelium of the duodenum and also with an irritation of the gall bladder. Intense infections in children are considered by some to cause dysentery, celiac syndrome, or a sprue-like condition.[31] The parasites and the associated intestinal symptoms disappear following treatment with atabrine.

Trichomonas vaginalis is a cosmopolitan common parasite of the vagina and male genital tract. Infection is commonly symptomless but may produce in the female a severe vaginitis and in the male may occasionally be associated with an urethritis.[129] Transmission occurs primarily through sexual intercourse as the parasite has no resistant stage. Variation in virulence is related to strain of the parasite. It is interesting that infected individuals also are usually infected with the nonpathogenic buccal flagellate, *Trichomonas tenax.*[20]

Flagellates related to the above such as *Trichomonas foetus* may be highly pathogenic. The latter, also transmitted by coitus, causes contagious abortion in cattle. *Trichomonas gallinae,* a common parasite of the domestic pigeon, has virulent pathogenic strains producing

lesions in the upper digestive tract and associated structures. Virulence transformation of avirulent strain forms treated with homogenates of pathogenic strains has been reported for this species.[64]

Chilomastix and Giardia are spread by ingestion of material contaminated with human feces containing their cysts. Trichomonas has no resistant cyst but the trophozoite may remain viable for more than an hour on dry fomites.

HEMOFLAGELLATES (MASTIGOPHORA)

The flagellate protozoa occurring in the blood stream or as intracellular parasites of man are termed hemoflagellates. All are members of the family Trypanosomidae with seven species found in man, *viz., Trypanosoma rhodesiense, T. gambiense, T. cruzi, T. rangeli, Leishmania donovani, Leishmania tropica,* and *L. braziliense.* All have probably evolved from parasites of the intestinal tract of insects and those parasitizing man still require blood-sucking insect vectors or intermediate hosts for normal transmission.[47] During their life cycles hemoflagellates display a variety of morphological types in regular stages. Since these types have been associated with particular genera the stages are named for the genera they resemble. As indicated in the accompanying figure these are the trypanosome, crithidial, leptomonad and leishmanial forms. The trypanosome form is characterized by having a flagellum arising from the posterior of the cell, attached to the cell by an undulating membrane running the length of the body, and ending anteriorly as a free flagellum. In the crithidial form the flagellum arises just anterior to the nucleus with the undulating membrane being correspondingly reduced. The flagellum in the leptomonad stage originates near the anterior end of the body with no associated membrane while in the ovoid leishmanial stage no free flagellum is present. The structures associated with the point of origin of the flagellum, the kinetoplast and basal granule, may still be seen in the leishmania and are of diagnostic importance.

The medically important hemoflagellates fall into three groups in two genera. *Trypanosoma gambiense* and related forms exhibit the trypaniform stage in the vertebrate host and trypaniform and crithidial stages in the invertebrate. *T. cruzi* exhibits all four stages in the vertebrate host, trypaniform and crithidial stages in the invertebrate. Members of the genus Leishmania exhibit only the leishmaniform stage in the vertebrate and the leptomonas stage in the invertebrate host.

Reproduction of all forms is by binary

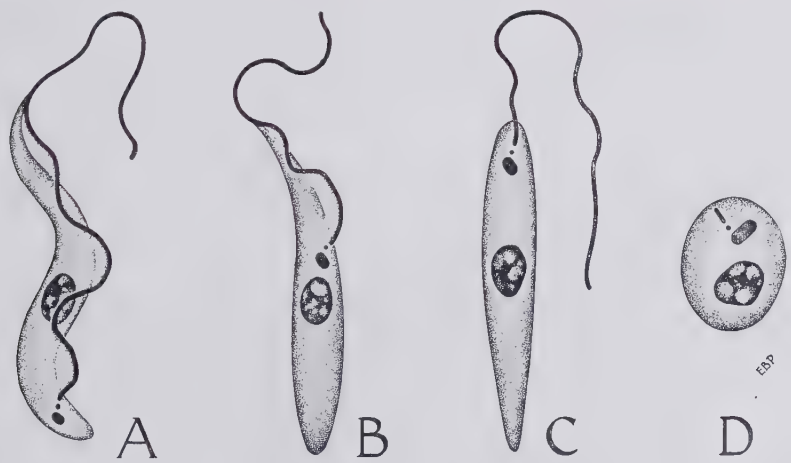


Figure 234. Morphological types of hemoflagellates. *A,* Trypanosome stage. *B,* Crithidial stage. *C,* Leptomonas stage. *D,* Leishmania stage.

fission. The nucleus, blepharoplast, and parabasal body divide, a new flagellum arises from one blepharoplast, and the cytoplasm divides longitudinally to produce two daughter cells. In the species with which we are concerned, the leishmaniform stages are intracelluar in the vertebrate host. The flagellated stages inhabit body fluids of vertebrates or the alimentary tract of insects.

The Trypanosomes

Trypanosoma Gambiense. Ford observed, in the blood of a Gambian native, a flagellate which was described by Dutton in 1902 as *T. gambiense*. In 1903, Castellani observed similar flagellates in the cerebrospinal fluid of a sleeping sickness patient in Uganda. Bruce and Nabarro, in 1903, transmitted *T. gambiense* to monkeys with a tsetse fly, and Kleine, in 1909, showed that the parasites underwent a cyclic development in the fly.

Characteristics and life cycle. In the blood and lymph of early cases of African sleeping sickness *T. gambiense* exhibits the trypaniform stage, which in fresh preparations may be seen wriggling among the erythrocytes. It measures 15 to 40 μ in length, varying from short, broad forms with no free flagellum beyond the undulating membrane to long thin forms with a free flagellum. Reproduction, as in all members of the family, is by binary fission. The transmitting insect, a tsetse fly (Glossina), becomes infected by ingestion of the parasites in infected blood. In the stomach of the fly those flagellates which survive multiply as trypaniform and crithidial stages, first in the crop, stomach, and intestine and later in the salivary glands. Here they form infective parasites, which are injected by the bite of the fly 20 days or more after its infecting blood meal.

On NNN medium, a concentrated blood agar, the parasites multiply to a limited extent. Continuous cultivation has been achieved in some instances, but it is too difficult to be of practical value. Abundant multiplication occurs on the chorioallantoic membrane of the developing chick embryo. Development of a related species, *T. vivax*, to the infective stage in tsetse fly tissue culture has been accomplished.[128]

African sleeping sickness. The incubation period following an infective tsetse fly bite varies from 2 or 3 weeks to several months. The first stage of the human disease is a systemic infection in which the parasites are found chiefly in the blood but also in the lymph. Irregular fever is generally the first symptom. Somewhat later, as the parasites come to predominate in the lymph nodes, these organs and the spleen are enlarged, and anemia and wasting are seen. Cardiac injury may be prominent, and edema is often present. This stage gradually gives way to the sleeping sickness stage, in which the parasites are most abundant in the cerebrospinal fluid, occurring less commonly in the lymph. The central nervous system exhibits the prominent lesions, typically resulting in somnolence, apathy, and weakness, less commonly in mania and

Figure 235. *Trypanosoma gambiense* in stained blood film; × about 2000. (Krall.)

other violent manifestations. Coma and death are the final outcome. Typically the course is of several months' duration, but in parts of East Africa the disease develops more rapidly, invasion of the central nervous system occurs early, and death frequently results from cardiac injury before the typical sleeping sickness stage is reached. In the blood of laboratory animals infected from cases in this area about 5 per cent of the flagellates are short, showing posterior nuclei; this is true only rarely in the parasites from other areas. Because of these differences in morphology and pathogenicity and the fact that transmission involves different species of tsetse flies, Stephens and Fantham separated the East African parasites as a different species, *T. rhodesiense*. Most authors now consider this a local race of *T. gambiense*.[32]

Immunity. Spontaneous recovery is said to occur occasionally in African sleeping sickness. Except for this there are no data available on natural or acquired immunity in man. In rats and mice *T. gambiense* and related parasites produce rapidly fatal parasitemia, while in guinea pigs and some other hosts they show a characteristic relapsing type of infection. After a period of unchecked multiplication, the organisms are rapidly destroyed, only to reappear in a few days and repeat the cycle. An antibody arising in the serum of a guinea pig at the time of crisis destroys parasites of the strain present before the crisis, while the strain present after flagellates have reappeared is resistant to this antibody. This phenomenon has been observed in animals infected originally with a single parasite, indicating that the change in antigenic structure cannot depend on selection alone but must involve adaptive modification of the parasite.

Diagnosis. Various serum reactions are observable in African trypanosomiasis but are not dependable for diagnosis, chiefly because of the antigenic lability of the parasites. Laboratory diagnosis depends on the finding and morphological identification of the organisms in blood, lymph node juice, or cerebrospinal fluid. Dry smears stained with blood stains, such as Wright's, are used, either of the whole fluid as obtained or of centrifuged specimens. In the case of blood, thick or thin films may be used. The parasites are usually scarce, and a negative finding in any one of the body fluids is of little significance.

Epidemiology and control. Rare instances of probable venereal infection have been reported. However, the normal transfer of infection occurs by the bite of tsetse flies as described above. Two species of flies are of most importance, *Glossina palpalis* in West Africa and *G. morsitans* in East Africa, where the more virulent Rhodesian form of the disease occurs. Other species of importance are *G. tachinoides, G. swynnertoni, G. pallidipes,* and *G. austeni.* These insects are relatives of the common housefly. They are limited to tropical Africa and a small area in South Arabia, and the human disease is confined to their range. They resemble the housefly in appearance except for the long, narrow proboscis, which is held straight forward from the head, and the manner of folding the wings at rest, flat on the back with one directly above the other. Both males and females bite and can transmit the disease. They bite exclusively by day. The larvae develop completely in the body of the female, are deposited singly on loose soil or sand in well shaded places, and quickly burrow into the soil to pupate. After 4 to 8 weeks the adults emerge. *G. palpalis* breeds almost entirely near water and is thus more limited in local distribution than *G. morsitans,* which is less dependent on shade and moisture.

The significance of wild animals in spread of African sleeping sickness has long been disputed. Various large wild animals, especially the sitatunga antelope, harbor the flagellates, usually without symptoms, and they have been shown to maintain the infection for long periods in the absence of human reservoirs. However, most authorities agree that man is usually the source of infection. The incidence of human infection varies widely. It is not commonly higher than 2 per cent at present, though in the

past villages were observed with infection rates as high as 50 per cent, and catastrophic epidemics have occurred.

The variety of control methods in use testifies to their relative ineffectiveness. Several valuable drugs are available for treatment of the human infection, a method whose success is proportional to the local significance of man as a reservoir of infection. Tryparsamide and other arsenicals are effective in all stages of the disease, though less so in the Rhodesian form, where Bayer 205 (suramin) is more active. This drug is useless in the sleeping sickness stage of the disease but is effective during the first stage and has the particularly valuable property of protecting against infection for at least 3 months after administration. A new series of diamidine compounds represented by pentamidine shows genuine promise. Pentamidine cures existing cases and protects against infection for at least 6 months after administration. Hundreds of thousands of African natives have received this drug in mass treatment campaigns, and the resulting reduction in incidence of infection suggests that this is the most effective control measure available.

In some areas mass destruction of the reservoir game animals has been attempted, but the success of this measure is doubtful. Where the human and animal infection rates are particularly high, wholesale removal of human populations has been carried out. Control of migrants, a perpetual problem in much of Africa, is utilized in some districts in an effort to minimize spread of the disease.

The life cycle and complex behavior of the tsetse flies make their control exceedingly difficult. Traps and hand-catching have greatly reduced the fly population in some regions. Inspection and fumigation of vehicles have been used to limit the spread of tsetse. The best single method is clearing of forest and brush, particularly along streams and around villages, which destroys the breeding and resting places of the flies.

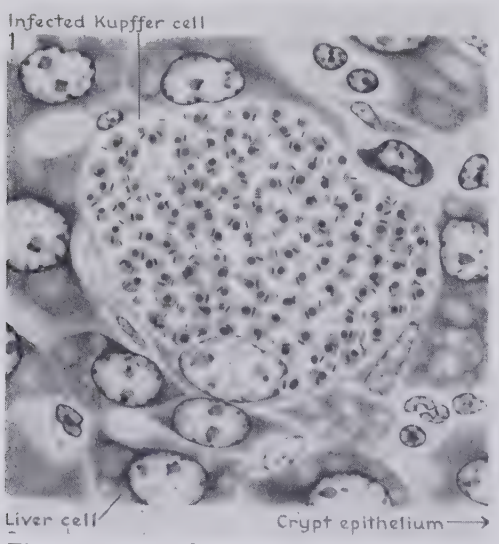


Figure 236. Leishmaniform stage of *Trypanosoma cruzi* Tulahuén strain in Kupffer cell of liver. (Taliaferro and Pizzi.)

Trypanosoma Cruzi. Chagas, in 1909, discovered intermediate stage flagellates in the hind gut of the bug *Triatoma megista* in Brazil. He showed the infectivity of these flagellates for mammals and later found trypaniform stages in children with a characteristic disease, now known as Chagas' disease. He described the organism as *Schizotrypanum cruzi*, but it is now called *T. cruzi* by most authors.

Characteristics and life cycle. The parasites as seen in the blood of early cases of Chagas' disease are of the trypaniform type, fundamentally similar to those of *T. gambiense* but smaller, about 20 μ in length, and with the posterior end pointed. No multiplication occurs in the blood. The predominant phase in man and experimental animals is a small, rounded nonflagellate stage, 3 to 5 μ in diameter, occurring in clumps in various tissue cells—heart muscle, macrophages, and endothelium. These organisms have a nucleus and parabasal body as described above. They multiply by binary fission, producing dense clumps of organisms which fill the infected cells. Small numbers of trypaniform stages are apparently continually produced and shed into the circulating blood from the nests of intracellular nonflagellate parasites.

Various bugs of the family Redu-

viidae acquire the infection from a blood meal containing the trypaniform stages. These become crithidial flagellates in the midgut and hindgut, where they multiply to produce infective trypaniform flagellates 2 to 3 weeks after the infecting blood meal. These organisms are shed in the feces of the bug. They infect man by entering the wound caused by the bite of the infected bug or by penetrating the mucosae, particularly of the mouth or eye.

Continuous culture of *T. cruzi* on NNN medium and in simpler broth mediums is relatively easy. The parasites multiply as trypaniform and, predominantly, crithidial flagellates. The nonflagellate stages have been grown in tissue cultures of macrophages from infected animals.

Chagas' disease. Chagas described the infection from a region having serious endemic goiter, and most of his cases had marked thyroid pathology. It has since been shown in other regions that the goitrous manifestations he described are not part of Chagas' disease. The incubation period is 1 to 2 weeks. Irregular fever and edema, particularly of the eyelids (Romaña's sign), characterize the acute phase, and there is considerable enlargement of lymph nodes, spleen, and liver toward the end of this period. The acute disease is rare except in small children, in whom occur almost the only deaths attributable to the infection. The chronic disease, which occurs in adults or following the acute stage in children, varies in symptomatology with the localization of the parasites but is most often characterized by myocarditis.[104]

In addition to the myotropic strain, a reticulotropic strain (Tulahuén) occurs in Chile. Enlargement of the spleen and liver as well as engorgement of bone marrow may give a pathology resembling visceral leishmaniasis. Transplacental infection may occur but rarely and the conditions of megacolon and megesophagus found in Argentina and Chile are attributed to parasitism by this species.

Immunity. Evidence concerning acquired immunity is lacking in the human disease, but experimental animals are immune to reinfection after recovery. Serum from such animals partially protects against experimental infection. The serum of human cases fixes complement in the presence of extracts of infected tissues or cultures of *T. cruzi,* and the complement-fixation test (the Machado reaction), or any of several modifications of it, is widely used for laboratory diagnosis. A relatively nonpathogenic strain[52] of *T. cruzi* has been found to occur as a parasite of wild mammals in Maryland.[136] Infection of mice with this strain confers immunity to superinfection with the more virulent strains.[75]

Diagnosis. Laboratory methods for diagnosis other than the complement fixation reaction depend on the demonstration of the parasites. During the early stages of the human disease trypaniform flagellates may be found in stained blood smears. Later, indirect evidence of the presence of parasites may be obtained by blood culture or by "xenodiagnosis" (host diagnosis), in which laboratory-reared bugs become infected after feeding on a case. Fluorescent antibody methods, although not in widespread use, have also been shown to be of practical diagnostic value.

Epidemiology and control. Infection is normally acquired from infected bugs as described above, although occasionally the disease is acquired by direct contamination of mucosae, as in the congenital infection of infants. The chief vectors are *Triatoma* (*Mestor*) *megista* and *Rhodnius prolixus,* but about forty species of the family Reduviidae have been incriminated. These insects are members of the order Hemiptera, or True Bugs, to which belong also bedbugs and many others. They are large insects with an elongated, cone-shaped head. Most species are predatory on other insects, but some live on vertebrate blood. They inhabit the nests of various animals and may occur in human houses of poor construction, where both the male and female insects commonly bite sleeping persons about the

mouth or eyes. The total life cycle, involving egg, larval, nymphal and adult stages, occupies 6 to 10 months.

The human disease is widespread in South and Central America but of low incidence in most areas. Various mammals are susceptible, and the infection occurs naturally in many of these, particularly dogs, cats, wild rodents, opossums, and armadillos, all of which serve as reservoirs of human infection. In the southwestern United States natural infections occur in the insects and in the wild rodents which they infest, but only one human case has been reported in this country. As mentioned above, a strain has also been isolated from raccoons in the eastern United States.

Successful control of Chagas' disease has not been attained. The most promising methods involve destruction of infected domestic animals and improvement of human houses to exclude the insects and the reservoir hosts. There is no successful chemotherapy for Chagas' disease, the drugs useful in African trypanosomiasis being inactive in this infection.

Trypanosoma Rangeli. Tejera, in 1920, described from a vector of *T. cruzi* in Venezuela a flagellate which he named *T. rangeli.* In 1942 this was obtained from humans by xenodiagnosis and since that time has been reported widely from Central and South America in the areas in which *Rhodnius prolixus,* its vector, occurs. Trypanosome division takes place in the peripheral circulation of man, dog, and monkeys with no leishmaniform stages demonstrated. No clinical manifestations are known and two experimental infections in man were without symptoms. In contrast to *T. cruzi, T. rangeli* is transmitted to man through the bite of the insect.

Other Species of Trypanosoma. A number of important diseases of domestic animals are caused by species of Trypanosoma similar to *T. gambiense. Nagana* is a rapidly fatal disease of the horse family, and to a less extent of cattle and dogs, occurring in a wide area of East Africa. It is caused by *T. brucei,* an organism very similar to the Rhodesian form of *T. gambiense.* A number of other species transmitted by tsetse flies are important disease agents of animals in Africa. *T. evansi* causes a disease of horses, camels, and mules known as *surra,* which is widespread in Asia, extending to Russia, Arabia, and Madagascar.

T. lewisi of rats elicits an interesting antibody, which inhibits reproduction of the parasites without killing them.[125] This antibody, ablastin, interferes with nuclear division and the production of nucleoprotein by the parasite.[102, 124]

The Leishmanias

Leishmania Donovani. Leishman and Donovan, in 1903, described oval parasites of the macrophages in cases of kala-azar in India. These were recognized as mastigophora when Rogers showed they developed into motile flagellates in culture.

Characteristics and life cycle. In the human disease, kala-azar, the parasites occur as nonflagellate stages, 3 to 5 μ long, in macrophages, where they resemble the nonflagellate stages of *T. cruzi* as shown in the accompanying figure. They multiply by binary fission until the cytoplasm of the host cell is crowded, when they escape to infect new cells. While the parasites predominate in internal organs, they occur in the skin macrophages as well, and it is probably from this site that the intermediate hosts, sandflies of the genus Phlebotomus, become infected. The parasites transform into the leptomonas stage, 14 to 20 μ long, and multiply in the midgut and foregut of the insect, which becomes infective after a week or more. The flagellates, injected by the bite of the fly into a new host, re-establish the vertebrate phase of the cycle.

L. donovani is easily cultivated in NNN and other mediums at 22° to 35° C., multiplying in the leptomonas stage as in the insect host. In tissue cultures of spleen from infected animals the nonflagellate stages multiply abundantly.

Figure 237. *Leishmania donovani*, free and in phagocytes, in Giemsa stained impression smear of spleen. Camera lucida. Approx. × 2000.

Kala-azar. Visceral leishmaniasis, or kala-azar, is usually a chronic disease.[83] Typically it begins, after an incubation period of 1 to 4 months (sometimes much longer), with a high temperature, followed by an irregular fever. The spleen and liver enlarge greatly with hyperplasia of the parasitized macrophage system. Wasting, emaciation, and edema are common. Dysentery often occurs as a result of heavy infection in the intestinal wall. The skin is typically dusky in hue, whence the name kala-azar, meaning "black fever." The skin is infected with the parasite but usually does not show lesions until months after systemic recovery when depigmented areas appear, later often becoming slightly raised papules. Anemia and leucopenia are characteristic. Adrenal insufficiency has been reported. Death is the rule in untreated cases, usually as a result of secondary infection.

Immunity. Treated or spontaneously cured kala-azar is apparently followed by a lasting, solid immunity, for second infections are exceedingly rare. However, vaccines have failed to protect against or ameliorate the disease.

Diagnosis and Treatment. Crucial laboratory diagnosis of kala-azar may be obtained by the finding of parasites in biopsies of skin, spleen, liver, or bone marrow. Sternal bone marrow puncture is a reliable and safe procedure. Blood cultures may be positive. In addition, a group of nonspecific serological reactions has shown great value. These reactions depend on the fact that the serum euglobulin is greatly increased in amount in cases of kala-azar. In the formol-gel test (Napier's aldehyde reaction), positive serums form an opaque gel when formalin is added. In the antimony test a pentavalent antimonial drug causes a heavy flocculent precipitate in serums of cases. These tests are positive in over 80 per cent of cases and give false positives only occasionally with other diseases, especially schistosomiasis and malaria.

Several antimony compounds, particularly Neostibosan and Solustibosan, are effective in kala-azar. Successful treatment is often followed by dermal leishmaniasis, which may persist for several months. The drugs stilbamidine and pentamidine mentioned in connection with African sleeping sickness are also effective in kala-azar.

Epidemiology and control. Kala-azar has been reported in China, India, South

Russia, Mesopotamia, the Mediterranean littoral, southern Arabia, Equatorial Africa, and parts of South and Central America. In Asia it is apparently a disease of man, though various lower animals, particularly dogs, hamsters, and mice, are susceptible. Infection may occur at any age but is most common in older children and young adults. In the Mediterranean area, however, it is primarily a disease of small children. Here dogs are commonly infected and apparently serve as the reservoir, since, unlike children, they show abundant parasites in the skin. The Mediterranean parasite is called by some authors *L. infantum,* and the South American form has been described as *L. chagasi,* but both are generally believed to belong to the species *L. donovani.*

Factors of seasonal and geographical distribution early pointed to sandflies as probable vectors, and it was soon shown that they were susceptible, developing heavy intestinal infections when fed on cases. In these flies the pharynx often becomes blocked with the simple flagellate stages, and in their vigorous efforts to feed, the "blocked" flies apparently are more likely to inject parasites.

The sandfly vectors belong to the genus Phlebotomus of the family of small flies, Psychodidae. The most important species are *Phlebotomus argentipes* in India, *P. chinensis* in China, and *P. major* and *P. perniciosus* in the Mediterranean region. The vectors in other areas are as yet unknown. The adult flies are minute, night-biting insects. They fly weakly and travel very short distances but penetrate ordinary window-screening with ease. The larvae develop in loose damp soil or debris, mostly in cracks in walls, cliffs, caves, etc., the entire life cycle requiring 1 to 2 months.

The transmitting insects can be controlled by cleaning up potential breeding places or by spraying them with DDT. Treatment of human cases is also widely used for control of the spread of kala-azar.

Leishmania Tropica. *L. tropica* was discovered by Wright, in 1903, in an Armenian patient in Boston. Morphologically and culturally identical with *L. donovani,* it differs in infecting primarily the skin, where it proliferates in the macrophages of the subcutaneous tissue. In man, dogs, and wild rodents it produces large single or multiple ulcers, usually of exposed parts of the skin, known as oriental sore. These lesions appear after an incubation period of 10 days to several months. They increase to a diameter of 1 to 3 cm. and heal spontaneously after several months to leave a disfiguring scar. Permanent immunity follows infection, and deliberate induction of sores on unexposed parts of the body has long been practiced in the Middle East to avoid the disfigurement of exposed scars. Transmission can be effected by direct contact, fomites, and houseflies, but the principal spread is undoubtedly by sandflies in a manner similar to kala-azar. Oriental sore occurs in Southern Asia, Southern Russia, the Near East, Equatorial Africa, and the Mediterranean region, the principal vectors being *P. sergenti, P. papatasii,* and, probably, *P. caucasicus.* Laboratory diagnosis is made by identification of the parasites in stained smears of scrapings from the lesions. Reliable diagnosis of oriental sore has been reported with an intradermal test. Since it is often very difficult to detect parasites in the lesions, such a procedure would be valuable. Local or systemic administration of antimony compounds such as Neostibosan is usually effective in treatment.

Leishmania Braziliensis. Morphologically this species is indistinguishable from *L. tropica* and *L. donovani* and its primary lesion closely resembles that of *L. tropica.*[49] Superficial granular or moist ulcers may occur at any skin site. In some endemic areas the parasite characteristically migrates to secondary foci, particularly mucocutaneous junctions, causing extensive destructive erosion of the nasopharynx, larynx, and associated structures, with facial disfigurement. Metastatic lesions occur with probable spread through the lymphatics and leishmanias have been recovered from the peripheral blood.

Diagnosis and treatment are the same as used for the other leishmanias, by recovery and identification of the parasite in stained smears from lesions. An intradermal test, the Montenegro reaction, utilizing an antigen derived from cultured leishmanias is also of value. The disease is known by a variety of names throughout its range, including uta, espundia, chiclero ulcer, and mucocutaneous leishmaniasis and has a distribution ranging from Argentina north into the Yucatan peninsula of Mexico.

SPOROZOA

Malarial Parasites. With the exception of an uncommon intestinal parasite, the only sporozoa infecting man are the malarial parasites. They were first recognized by Laveran in 1880, and the life cycle in human erythrocytes was described by Golgi. Manson's suggestions led Ross to the demonstration in 1898 of mosquito transmission of avian malaria, and Grassi, Bignami, and Bastianelli later in the same year showed the mechanism of spread of human malaria. Man harbors four species of malarial parasites, of which *Plasmodium vivax* will serve as an example.[14]

Characteristics and life cycle. In fresh preparations of infected blood the parasites of *P. vivax* appear as clear areas in the erythrocytes. They contain yellow or brown granules of pigment, a digestion product of hemoglobin which has been identified microchemically as hematin. In blood films colored with stains such as Giemsa the parasites show blue cytoplasm and violet red nuclei.[43] The earliest stage in the erythrocyte, the *ring* stage, consists of a thin ring of cytoplasm with a nucleus at one side. The parasite grows, becoming an irregular uninucleate body containing several brown pigment granules. This stage is known as the *ameboid trophozoite*. The parasitized cell has now enlarged somewhat and may show scattered throughout its cytoplasm minute red granules, "Schüffner's dots," which are apparently a result of injury to the cell. The parasite continues to grow, phagocytizing the erythrocyte hemoglobin which it digests, thereby accumulating more pigment. Eventually it nearly fills the erythrocyte, which is now about one and one-half times its normal diameter. The nucleus divides repeatedly until twelve to twenty-four, usually about sixteen, nuclei are present. This is the *schizont* stage. Finally, in the *segmenter* stage, the cytoplasm divides, a portion surrounding each nucleus. The pigment is left in a dense clump, and the cell disintegrates to release the daughter cells, or *merozoites,* into the plasma. Here they invade fresh erythrocytes and repeat the cycle. The above process of growth and multiple fission is known as schizogony. It occupies, in *P. vivax,* about 48 hours, and the growth is regulated by the daily cycle of activity of the host, so that segmentation usually occurs at about the same time every other day.

After several schizogonic cycles a difference may be noted in the infection. Some ameboid stages, instead of becoming schizonts and undergoing asexual reproduction, develop into large uninucleate parasites with scattered pigment granules. These are the sexual stages. The female, or *macrogametocyte,* shows a compact, dark red nucleus and intense blue cytoplasm. The male, or *microgametocyte,* has a more diffuse, less deeply stained nucleus, and the cytoplasm is paler, often pinkish rather than blue. The gametocytes undergo no further development in man, eventually degenerating or being destroyed unless they are taken up by a susceptible mosquito.

In the stomach of the mosquito, gametes are produced. The macrogametocyte escapes from the erythrocyte and becomes a single *macrogamete,* corresponding to a metazoan ovum. The microgametocyte produces at its surface, by a process generally called "exflagellation," four to eight long, whip-like *microgametes,* counterparts of the spermatozoa of higher animals. One of these actively wriggling microgametes ferti-

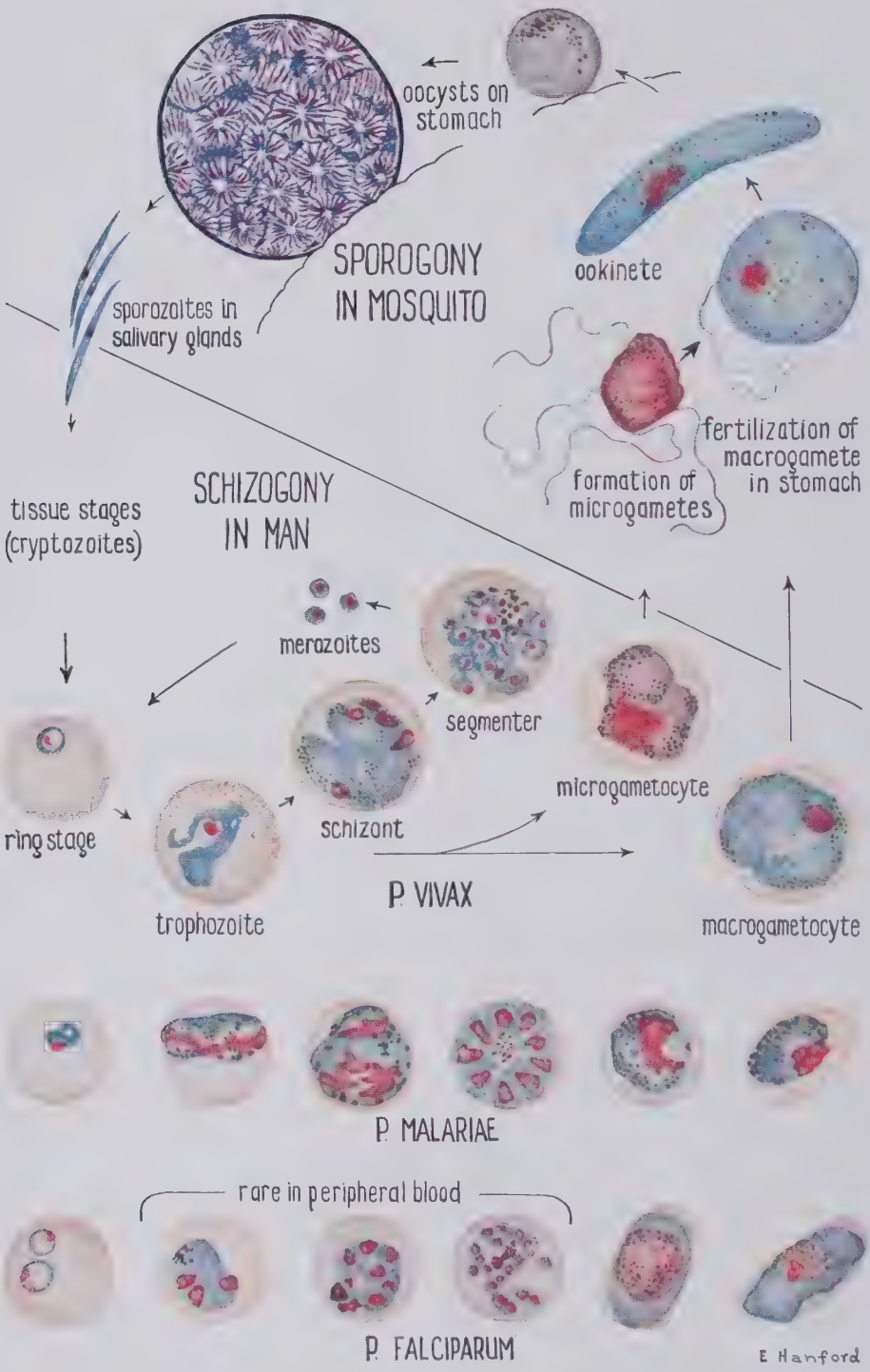


Figure 238. Life cycle and comparative morphology of the malarial parasites of man. (Schizogonic stages redrawn from Huff, *Manual of Medical Parasitology.*) Oöcysts × 600; remaining stages × 2000.

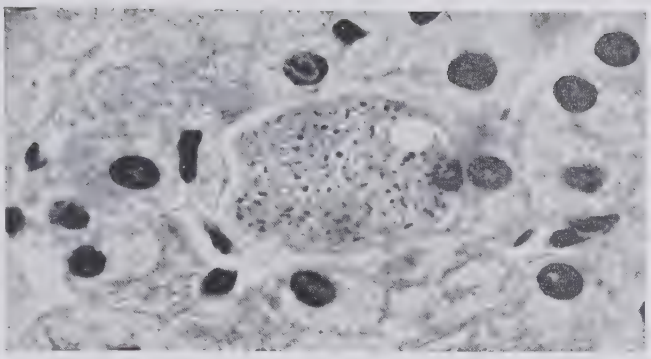


Figure 239. Cryptozoite of *Plasmodium vivax* in liver section. (Courtesy of Col. H. E. Shortt.)

lizes a macrogamete. The resulting spherical zygote on the surface of the blood meal becomes entrapped between the contracting epithelial cells of the mosquito stomach as the meal is digested. Growth results in an oöcyst which comes to rest on the outside of the stomach. (The fusiform oökinete usually described as resulting from the zygote and producing the oöcyst is in all probability a degenerating stage.)* The above process in the mosquito occupies 1 to 2 days. Nuclear multiplication occurs and the oöcyst grows until a diameter of about 50 μ is attained. The oöcyst now contains many hundreds of nuclei. Each acquires a bit of cytoplasm and becomes a spindle-shaped body, about 8 μ long, the *sporozoite*. With the rupture of the oöcyst, these sporozoites scatter throughout the body of the mosquito. Many accumulate in the salivary glands, where they are injected into man by the biting mosquito. The complete development in the mosquito requires from 1 to 2 weeks; 25° C. is said to be the optimal temperature, no development occurring below 15° C. nor above 30° C.

For many years it was believed that the sporozoites entered erythrocytes to initiate the schizogonic cycle described above. Indirect evidence suggested that the parasites underwent a different type of development before invading the blood. This development was first discovered in a malarial infection of birds.[67] The sporozoites were found to enter fixed tissue cells where, as *cryptozoites,* they undergo a type of schizogony fundamentally like that in erythrocytes except that no pigment is produced. Segmentation produces merozoites which invade new macrophages and repeat the cycle. Some of the second generation merozoites enter erythrocytes to establish the blood schizogony. Others continue to reproduce in tissue cells as *exoerythrocytic* stages, probably throughout the whole course of infection, and may repeatedly give rise to new schizonts in erythrocytes. The cryptozoites and later exoerythrocytic stages are resistant to drugs effective against the blood parasites.[15]

Until 1948 neither cryptozoites nor later exoerythrocytic stages had been observed in mammalian malarias. They were observed first in monkey malaria and later in a human volunteer infected with enormous numbers of sporozoites of *P. vivax*. Large schizonts were found in the liver 5 to 10 days after sporozoite inoculation. Unlike those in avian malarias, which occur in macrophages, the cryptozoites in *P. vivax* were said to be in hepatic cells. The parasites observed appear to constitute a second generation of reproduction, but no organisms have been observed before the fifth day of infection. In one monkey schizonts have been observed in the liver 3½ months after infection, indicating persistence of the tissue parasites during and after the acute blood infection. Knowledge of various aspects of the exoerythrocytic cycle of plasmodia of all vertebrates is

* Howard, L. E.: Studies on the mechanism of infection of the mosquito midgut by *Plasmodium gallinaceum*. Amer. J. Hyg., 1962, 75:287–300.

fully covered in a monograph by Bray[15] and the more recent literature is covered by Eyles.[37]

While much study is still required in this very important aspect of malaria, it is now possible to reconstruct in essence the development of infection in *P. vivax.* Sporozoites initiate a type of schizogony similar to that described above for avian malarias. In *P. vivax,* however, at least part of this cycle seems to be in cells other than macrophages. After about 9 days some of the merozoites initiate the erythrocytic schizogony. Others apparently continue the exoerythrocytic infection, which can later give rise to new generations of erythrocytic parasites.

Limited success has been achieved in the artificial cultivation of malarial parasites. Geiman observed several generations of reproduction with *P. vivax* in erythrocytes *in vitro.*[50] An avian plasmodium has since been maintained for ten passages over a period of 18 days. Some growth has also occurred in a noncellular medium containing a lysate of erythrocytes.[127] A good deal of physiological information has come from these studies, but practical cultivation for routine work has not been obtained. Exoerythrocytic stages of avian malarias have been grown in tissue cultures of macrophages, and various cells derived from chick embryo heart, and chick meninges. Phase photocinematography of cultures are showing hitherto unsuspected morphological features of the exoerythrocytic schizonts and merozoites as well as providing information on motility and the mechanisms of cell invasion.[68]

The life cycles and morphology of the other human malarial parasites in erythrocytes are fundamentally similar to those of *P. vivax. P. malariae* requires 72 hours for the completion of schizogony. The erythrocytic parasites show several differences of diagnostic value. No granules of the type of Schüffner's dots are seen in the infected cells. The cells are not enlarged during growth of the parasites. The older trophozoite is not ameboid like that of *P. vivax* but commonly exhibits a "band" form across the parasitized cell. The segmenter produces six to fourteen, usually eight, merozoites, arranged in a "rosette" form. The gametocytes are small, and the pigment typically occurs in abundant, large dark granules. The optimal temperature for sporogony in the mosquito is said to be about 22° C., and sporogony is slow, requiring 3 weeks or more.

P. falciparum, like *P. malariae,* causes no enlargement of the infected cells, but some parasitized cells show granules, Maurer's dots, which correspond to the Schüffner's dots of *P. vivax.* Schizogony results in six to twenty-four merozoites. The schizonts and segmenters typically accumulate in capillaries of internal organs, appearing in the peripheral blood only in very heavy infections. Normally, therefore, only ring stages and gametocytes are seen in blood films. Infection of erythrocytes by two or more rings is common and the rings often contain two nuclei. The gametocytes are distinctive, exhibiting a characteristic sausage shape which distorts or obliterates the infected cell. Because of their shape, they are commonly called "crescents." The pigment is finely granular or amorphous in appearance and in the gametocytes it is typically concentrated about the nucleus. The optimal temperature for sporogony is said to be 29° C.

P. ovale is a rare species showing similarities to both *P. vivax* and *P. malariae* but characterized chiefly by the fact that the infected cell is often distorted into an oval. Its significance is not known, although many investigators consider it an aberrant type of *P. vivax.*

Malaria. Vivax malaria, or *benign tertian malaria,* is featured by typical chills and fever occurring at the time of segmentation of the peripheral blood parasites. These paroxysms begin with an acute, shaking chill while the temperature is rising. A "hot stage" occurs at the fever peak, the patient feeling unbearably hot and the oral temperature usually reaching 104° or 105° F. This gives way to a "sweating stage" during which the fever falls rapidly to normal or slightly below. Parasite products or red

blood cell contents released at disruption of the infected cells are presumably responsible for the paroxysm. Paroxysms coincide, as stated above, with the time of parasite segmentation. Hence they occur every 48 hours if all the parasites reproduce on the same day. Often, however, distinct broods of parasites segment on alternate days. Vivax malaria commonly shows quotidian (daily) chills for several days followed by the suppression of one brood of parasites with resultant tertian chills (every other day). Between paroxysms the patient feels and appears normal.

Significant anemia occurs commonly in vivax malaria but is rarely serious unless other factors are involved. A week or more after the beginning of an attack, particularly in children, the spleen usually enlarges and remains enlarged for 2 to 6 months after termination of the attack. The untreated attack typically lasts 3 to 6 weeks, often with temporary cessations of clinical activity. It is followed by a period of latency during which parasites cannot be found microscopically in the peripheral blood. During part of this time large transfusions of blood from the infected person fail to induce infection in recipients. This suggests that exoerythrocytic stages are responsible for maintenance of infection during latency. In many cases renewal of clinical activity, or relapse, occurs after 2 to 10 months of latency, the clinical picture resembling that in the primary attack. The infection usually dies out after 2 to 3 years, and no further relapses occur.

Malaria due to *P. malariae* is known, because of the 72-hour cycle of reproduction, as *quartan malaria,* the chills occurring every third day. It is basically similar to vivax malaria, though the paroxysms are often more severe. The incubation period is usually long, 3 weeks or more, and the period of clinical activity typically lasts for several months. Relapses are uncommon, but latent infection may persist for many years, as shown by the occasional development of quartan malaria in recipients of blood transfusions from persons who have not shown evidence of infection for 30 years or more.

Falciparum malaria is widely known as *malignant tertian* or *estivo-autumnal malaria.* As noted above, *P. falciparum* is characterized by accumulation of schizogonic stages in internal organs. As a result, in addition to or instead of the typical paroxysms, which are quotidian or tertian, various local manifestations of the disease may be prominent. This is especially true in the tropics where such "pernicious" malaria may follow a series of typical chills. The most common types are algid (cold) malaria, gastrointestinal manifestations, and cerebral malaria with coma and often death. These peculiarities of falciparum malaria make it the cause of most of the deaths attributable to human malarial infection and an important factor in many deaths traced to other diseases. Blackwater fever, a dangerous hemoglobinuria, is associated with falciparum malaria and is thought to be an occasional late effect of repeated infection. *P. falciparum* infections rarely relapse and rarely persist more than a year or two. It is believed that the exoerythrocytic stages do not persist after the cycle in erythrocytes has begun.

Immunity. Malaria exhibits partial acquired immunity. After recovery from an acute attack, the individual is highly resistant to superinfection with a strain similar to that already present, but the resistance lasts for at most a few months after elimination of parasites from the body. This immunity depends on active phagocytosis and digestion of parasitized erythrocytes by the macrophages of spleen, liver, bone marrow, etc. That the parasitized cells are sensitized by antibodies is suggested by the fact that serum from recovered animals partially protects against infection. The immunity to superinfection is species- and strain-specific. During latency the parasites apparently continue to reproduce by exoerythrocytic schizogony, sporadically producing blood parasites which are held in balance by the acquired immunity. Relapse occurs when this balance is disturbed, permitting the blood para-

sites to increase greatly in numbers. The factors are little known, though it has been shown that operations increasing blood sugar induce relapse in avian malaria.

Different strains of vivax malaria exhibit characteristic patterns of activity. Thus, an American strain typically relapsed about 10 months after the initial infection, while a strain from New Guinea usually relapsed within 2 months. Data such as these indicate that reactivation of latent malaria is at least partly determined by inherent cyclic properties of the parasites. The lesser susceptibility to, as well as the lessened severity of infection of, the negroid race to *P. falciparum* and *P. vivax* is cited as an example of racial immunity (Chap. 9). Possession of the inherited sickle cell trait also confers a degree of immunity to *P. falciparum* as the erythrocytes are deficient with respect to the needs of the parasite. The maximum parasite count in those possessing the trait is much less than normal; the malaria mortality is less, resulting in the maintenance of a high population frequency of sickle cell anemia in some areas of *P. falciparum* endemicity.[92, 130]

Partial inhibition of avian and monkey malarial infections has been achieved with killed vaccines. There is no evidence concerning human malaria, but, because of the nature of immunity to malaria, it seems unlikely that vaccination would be of practical value. Serum agglutination of parasitized cells or isolated parasites has been reported in experimental infections. It is species- or strain-specific. Complement-fixing antibodies occur in the serum and are group-specific, reacting with extracts of avian and simian malarial parasites as well as with those from man.

Diagnosis. Laboratory diagnosis of acute malaria rests on the finding of parasites in peripheral blood films. They are most easily identified in thin films stained with Giemsa, but, since parasites are often scanty in the peripheral blood, dehemoglobinized thick films, similarly stained, are widely used. The rapid staining method of Field[43] is of especial value for such preparations. The fact that parasite morphology is abnormal in thick films is offset by the much greater volume of blood which can be examined in a comparable time. In surveys the characteristic spleen enlargement is a nonspecific but highly suggestive indication of current or recent malarial infection. Because it usually persists for several months it affords a more stable index of the infection rate in an area than do clinical or parasitological surveys.

Chemotherapy. Treatment of malaria for centuries depended on the bark or extracts of the bark of cinchona trees. The main active principle in cinchona bark is the alkaloid quinine. Quinine neither prevents nor cures the natural infection but rapidly suppresses the number of blood parasites below the density necessary to produce symptoms. The synthetic drug *quinacrine* (Atabrine) has similar action, and in addition it can cure falciparum malaria. *Chloroquine* and *Camoquin,* developed in large-scale World War II research, have properties like those of quinacrine but are more effective.[28] *Paludrine,* a product of British wartime research, is a very effective suppressive for vivax malaria and is both prophylactic and curative for *P. falciparum* infection. The distantly related *Daraprim* (pyrimethamine) has similar but stronger action. These two drugs suffer from the disadvantages that they act slowly and that the parasites may become resistant to them.

The above drugs taken continuously during exposure to vivax malaria act as suppressives, holding the infection down to subclinical levels. They do not prevent or cure the infection. *Primaquine,* however, destroys the exoerythrocytic stages and if given prior to invasion of the erythrocytes will prevent clinical malaria from occurring. It is also used in conjunction with drugs effective against the erythrocytic stages to cure established vivax infection, since elimination of the persistent exoerythrocytic stages is mandatory to prevent relapse.[4, 27]

An exciting "repository" chemotherapeutic agent has been tested in a small series of men exposed to sporozoite and blood induced *P. vivax* infection. A single intramuscular injection of this dihydrotriazine metabolite of chlorguanide (Cl-501) protects against repeated infection for a minimum of 7 months.*

As with other microorganisms, inheritable drug resistance can be induced in the plasmodia.[12, 56] Development of pyrimethamine resistance in both *P. vivax* and *P. falciparum*[143] has been observed on a number of occasions, and particularly virulent strains of Chloroquine-resistant *P. falciparum* have been recovered in Colombia and Thailand.[144] The former retained sensitivity to pyrimethamine but the latter is resistant to pyrimethamine but Atabrine sensitive.

Epidemiology and control. Malaria is the most common infectious disease of man, occurring throughout the warmer regions of the world and extending well into the temperate zones to occupy a large part of the land area between 60° N. and 40° S. latitude. The principal factor in distribution of the infection is climate, which affects both the distribution and abundance of the mosquito hosts and the development of the parasite in the mosquito. The human malarial parasites are not found in lower animals. Man is, therefore, the only reservoir of infection. Racial immunity is known in the case of *P. vivax.* Negroes are less readily infected with most strains than whites and seldom show symptoms. Local populations with high natural immunity to *P. falciparum* have also been reported. Age resistance is not known, but in endemic regions clinical malaria may be rare in native adults who have been constantly reinfected with the local strains of parasites.

Barring rare congenital cases, blood transfusions,[13] and accidental transfers of blood, as by drug addicts sharing contaminated syringes, human malaria is transmitted exclusively by the bites of certain mosquitoes of the genus Anopheles. This genus, comprising some 200 species throughout the world, is readily distinguished from the common house mosquitoes of the genera Culex and Aedes by several characteristics. The adult generally rests at an angle to the surface, with proboscis, head, and body in a straight line, whereas the others rest parallel, with the head and proboscis turned down. The wings are usually spotted, those of other mosquitoes being unmarked. The palps of the female are as long as the proboscis, giving the impression of three long appendages from the head in addition to the antennae, whereas the other genera have short, barely noticeable palps. The eggs bear inflated floats and are laid singly on the water, whereas in Culex they are laid in rafts on the water and in Aedes they are deposited singly on damp surfaces. The larvae of Anopheles lie flat at the surface of the water, feeding on floating particles, while those of other genera hang down in the water from an elongated breathing tube and usually feed on the bottom.

Only the female mosquitoes feed on blood, most Anopheles biting at dusk or in the night. The breeding places vary greatly in character, almost any type of water collection serving to support the larvae of one or more species of Anopheles. Most species, however, are quite specific in the types of water collections they choose. The principal malarial vector in the southeastern United States, *Anopheles quadrimaculatus,* breeds chiefly around the debris- and weed-covered edges of swamps, ponds, and sluggish streams. Important malarial vectors, however, are found in hill streams (*A. minimus* in Southern Asia), brackish marshes (*A. atroparvus* in Europe), small temporary pools (*A. gambiae* in Africa), water held in plants growing in the tops of trees (*A. bellator* in Trinidad), etc.

Not all species of Anopheles serve equally efficiently as vectors of malaria, about seventy-five being considered dangerous carriers in one or more regions. The chief determining factors are abundance, contact with man, and susceptibility. The last varies greatly among

* Thompson, P. E., Coatney, G. R., and Schmidt, L. H.: Unpublished observations.

species and among populations within species. Little is known of the factors in human malaria, but simple genetic differences have been shown to determine susceptibility of some Culex mosquitoes to avian malaria. In most situations which have been adequately studied, contact with man is the principal factor in the importance of species of Anopheles. Several susceptible species are abundant in the southeastern United States, but in most of this area only one, *A. quadrimaculatus,* shows sufficient preference for human blood to serve as an important carrier. In Europe species (formerly lumped together as *A. maculipennis*) distinguishable only by morphology of the egg differ so greatly in their relative preferences for human and animal blood that some are dangerous vectors while others, equally susceptible, are insignificant in the spread of malaria.

Malaria control rests largely on the interruption of the mosquito phase of the life cycle. This may be accomplished by reduction in mosquito numbers or prevention of contact between mosquitoes and man. If mosquito reduction is to be attempted, the differences in relative importance of different species, or even of the same species in different regions, require that the principal vectors in a given area be determined before control is attempted. Such determination is of value in avoiding waste of resources on unimportant mosquitoes. Furthermore, unplanned control efforts may completely miss the principal vectors and have even, on occasion, made matters worse by favoring the breeding of dangerous species. The "natural index" of infection, determined by examining stomachs and salivary glands of wild-caught female mosquitoes for oöcysts and sporozoites of malarial parasites, is the best guide to relative importance of a species. Unfortunately, the figure is often so low that dissection of large numbers of mosquitoes is required. In such cases expediency may justify indirect measures. Susceptibility may be determined by exposure of laboratory-reared mosquitoes to carriers and dissection of the fed females for stages of the parasite ("experimental index"). Contact with man may be measured by the location of resting mosquitoes (*e.g.,* in houses as against stables) or by precipitin tests on fed mosquitoes to determine the type of blood they contain.

If control is to be applied to the breeding of mosquitoes, the habits of the important vectors in an area must be determined. Elimination of breeding places is the method of choice in many regions. Water collections may be destroyed by drainage or filling. Salinity may be controlled by tide-gates. Breeding around the margins of reservoirs may be eliminated by water-level fluctuations. Shade may be increased or decreased by control of vegetation. Streams may be cleared or periodically sluiced. Specific methods, it will be obvious, depend on local conditions and the habits of the mosquitoes involved. Several effective poisons (larvicides) are available for attack on the aquatic stages. Oils sprayed on the water surface are toxic to the eggs, larvae, and pupae. DDT in oil is extremely effective. A minor method of local value is the stocking of ponds and pools with top-feeding minnows, Gambusia or Lebistes, which eat mosquito larvae.

Attack on adult mosquitoes, formerly a secondary measure, is now the most important technique for malaria control. The insecticides, DDT, lindane and dieldrin, have given malariologists a weapon of tremendous value. Sprayed on walls, screens, etc., they kill adult mosquitoes which rest on these surfaces, even several months after application. For those species of Anopheles which enter houses to bite man these insecticides have already demonstrated their value as an inexpensive and highly effective tool in malaria control. Malaria is no longer endemic in the United States and only sporadic cases of exotic origin are found today.[110] The success of the control of malaria here and in several other areas, along with the development of better chemotherapeutic agents and economically feasible residual sprays, has led to the hope that

world-wide malaria eradication will soon be an accomplished fact.[105] However, in addition to the factors previously known, the problem has become further complicated by the development of plasmodia resistant to chemotherapeutic agents and by the development of insecticide resistance in anophelene mosquitoes. At the end of 1958 only three resistant species were known and in the following year an additional 14 species of anophelene mosquitoes resistant to dieldrin and/or DDT were described.[91] More recently another complicating factor has been found in a vivax-type malaria of macaques transmissible to man. Accidental transmission via mosquito has been recorded and further experiments indicate its ability to produce clinical malaria in man. Two strains of this parasite, *Plasmodium cynomolgi,* have been transmitted from man to man by mosquito.[11, 29, 116]

Malarial parasites of lower animals. Malarial parasites fundamentally like those of man occur in apes, monkeys, bats, rodents, birds, and lizards. Since lower animal hosts are not available for the human malarial parasites, the natural parasites of monkeys, birds, and, recently, rodents have been widely used experimentally. The species most widely studied are *P. brasilianum, P. knowlesi,* and *P. cynomolgi* of monkeys, *P. cathemerium, P. relictum, P. gallinaceum,* and *P. lophurae* of birds, and *P. berghei* of rats and mice.

Intestinal Sporozoans

Two species of coccidia, *Isospora hominis* and *I. belli,* are sporadic infectors of man although cosmopolitan in distribution. A resistant oöcyst is passed in the stools and infected individuals have slight to severe diarrhea. No information on the life cycle, therapy or epidemiology is available and it is assumed that these species parasitize the epithelial cells of the small intestine as they do in the related species found in the dog and cat. The organisms have been described most frequently from the tropics and subtropics and have caused epidemics in military forces in the southwest Pacific islands during World War II.[42]

PARASITES OF UNCERTAIN AFFINITIES

Toxoplasma Gondii. *Toxoplasma gondii* was discovered in 1908 in rodents by Nicolle and Manceaux in Africa and by Splendore in Brazil. The first clearly recognized human case of disease due to this parasite was reported by Wolf and Cowen in 1937.[111, 138]

The organism is usually included with the sporozoa as a special group along with sarcocystis with which it shares some characteristics. Some authors question their inclusion within the protozoa. Reproduction is asexual with cysts or pseudocysts containing naked trophozoites. Only a single host is required (monoxenous) and locomotion is by gliding or flexing of the cell as no locomotor organelles are present.

Characteristics and life cycle. The trophozoite is a crescentic or oval body, 5 to 7 by 2 to 4 μ in size, staining blue with a reddish nucleus in Giemsa preparations as shown in the accompanying figure. It reproduces by binary fission in living cells. A great variety of cells are susceptible, but those chiefly infected are macrophages and other connective tissue cells, smooth and cardiac muscle, neurons, and microglia. While the parasites reproduce only in cells, they are often found free in body fluids.

In chronic infections they occur in a *pseudocyst* stage. This consists of a cluster of parasites resembling the trophozoites surrounded by an irregular wall. The origin of this wall is not known, but it may be the remains of a host cell in which the parasites proliferated. The pseudocysts are most abundant in the brain and skeletal muscle. They appear to be resting stages, more resistant than the reproductive forms.

Toxoplasma have not been cultivated in artificial mediums. They grow readily in tissue cultures of various types, in-

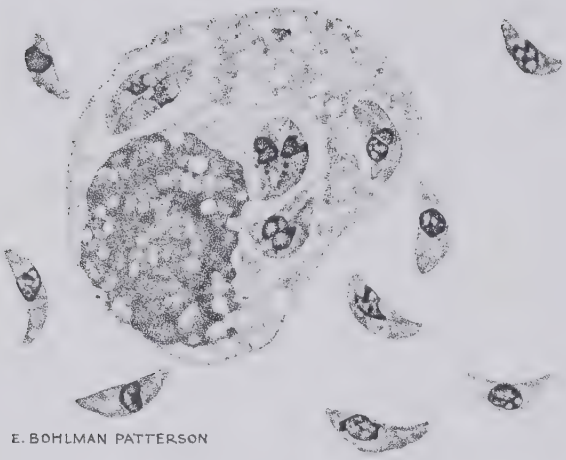


Figure 240. *Toxoplasma gondii,* free and in cell from mouse brain smear. Giemsa stain. Camera lucida. Approx. × 2000.

cluding HeLa cells, and in chick embryos.

Toxoplasmosis. Although infection with *T. gondii* is very common in man, it rarely gives rise to acute disease. Most of the clinical cases occur in newborn infants infected congenitally during late fetal development. The active lesions predominate in the central nervous system and eyes, causing blindness, gross defects of the brain, and, not infrequently, death. Infection of adults is usually subclinical, but pneumonitis, enlargement of lymph nodes and spleen, fever, and a maculopapular rash may be exhibited. A severe chorio-retinitis may result from infection of the eyes.[99]

Recovery is apparently accompanied by *immunity* to reinfection, although the pseudocysts, at least in lower animals, may persist for long periods in the tissues. Most experimental animals show solid immunity to reinfection. Monkeys and man exhibit a thermolabile antibody which protects rabbits against characteristic skin lesions resulting from the injection of toxoplasma.

Laboratory diagnosis requires isolation of the organisms or immunological tests. In neonatal cases it is usually possible to demonstrate the organisms by inoculation of laboratory animals. While all mammals and birds tested have been found susceptible, mice are preferred for isolation of the parasites because they are free from natural infection. Complement fixation may be used, but the most reliable immunological test is the dye inhibition reaction of Sabin and Feldman. This depends on the fact that toxoplasma in the peritoneal exudate of mice stain well with alkaline methylene blue in normal serum but not in immune serum. An accessory factor, present in fresh normal serum but destroyed by heat or by storage, is essential to the reaction. The test becomes positive 2 weeks or more after onset, reaching a titer of 1:256 or higher, and remains positive for many years. Fluorescent antibody methods have also been shown to be of diagnostic value.

Both acute and subacute toxoplasmosis have been treated with combinations of sulfadiazine and pyrimethamine. Treatment destroys the parasites in experimental animals and though beneficial in some human infections, toxic side effects may occur with irreparable damage to the brain and eye.[46]

Epidemiology. Perhaps the most vexing question occupying investigators of toxoplasmosis is the method or methods of natural transmission. It is clear that congenital infection of human embryos can occur, but it is equally clear that other routes of infection exist. The parasite is extremely abundant in nature. Surveys with the dye test show that up to 60 per cent or more of human populations have been infected. Investigations in various parts of the United States have revealed past infection rates of 17 to 35 per cent, with rates of over 65 per cent in the age group above 40. A wide variety of wild and domestic

birds and mammals show evidence of infection, often with high incidence. Human congenital infections have been associated with home environs in which mice and other vertebrates had abnormally high incidences of infection.[38] The pseudocysts in muscle are infective by mouth, and epidemiological evidence suggesting that infection might be acquired from undercooked pork has been reinforced by the finding of viable toxoplasma in samples of meat from 22 per cent of hogs from one slaughterhouse. However, orthodox Jews sometimes show evidence of a high incidence of infection, and some species of herbivorous animals are commonly infected, so it is obvious that pork or other meats are not the only important sources of infection. Toxoplasmosis should be regarded as one of the zoonoses in which man is only one of a relatively large number of natural hosts.

Sarcocystis. A single genus has been described with numerous species commonly found parasiting the muscles of mammals, birds, and reptiles. They are extremely common in ruminants occasionally producing a severe pathology in sheep. *Sarcocystis lindemanni,* the species reported in a number of instances infecting man, is not known to produce detectable symptoms. The organisms are fusiform tubes, round in section, up to several centimeters in length and usually in striated muscle. These "Meischer's tubes" have a hyaline wall. Internally they are divided into compartments containing many thousands of sickle-shaped individuals 12 to 16 μ in length. Experimentally, infection may be accomplished by ingestion of infected meat.

Pneumocystis Carinii. Infection with Pneumocystis is associated with highly contagious, epidemic, infantile, interstitial, plasma-cell pneumonia. The organisms are ovoid or crescent-shaped, 1 to 3 μ in diameter, and are commonly seen in rosettes of eight individuals within a membrane, free or within phagocytes. The organism is considered to be a protozoan and the etiological agent of the disease which in young infants frequently terminates in death. It has also been described from a wide variety of wild and domestic mammals. Although widely distributed, infection is but rarely diagnosed and no specific therapy has been reported.

THE METAZOA

All phyla of animals other than protozoa are commonly designated by the term Metazoa, and it will be convenient to mention certain general characteristics of the human parasites, mostly known as "worms," which belong to this aggregate. They are usually macroscopic in size, ranging from about a millimeter to several meters in length. Their structure is generally complex, and their life cycles vary from the simple production of infective eggs or larvae to complex alternation of generations involving as many as three different hosts. An important general characteristic is that multiplication usually does not occur in the human body, so that infection does not increase in intensity in the absence of re-exposure. Until recently true cultivation of worms parasitic in vertebrates had not been achieved. The great variety of metazoan parasites of man makes it impossible to discuss them extensively in a small space. Therefore, representative examples will be used wherever possible. For fuller discussions the reader is referred to the general texts cited earlier.

PLATYHELMINTHES

The phylum *Platyhelminthes,* or "flatworms," is differentiated from other phyla of animals by several character-

istics. They are bilaterally symmetrical and composed of the three primitive germ layers of tissues—ectoderm, endoderm, and mesoderm. The body cavity is not lined with mesoderm, as in higher forms, but filled with a spongy mass of cells, the *parenchyma.* The digestive tract is absent or, if present, lacks an anus, solid wastes being regurgitated through the mouth. There are three major classes—the Turbellaria, which are free-living forms or external parasites of aquatic animals, exemplified by Planaria; the Trematoda, or flukes, which are all parasitic; and the Cestoda, or tapeworms, also all parasitic.

Trematoda

The human lung fluke, *Paragonimus westermani,* and the blood fluke, *Schistosoma mansoni,* will serve as examples of the trematodes.

Paragonimus Westermani. The adult worm, found by Westerman in 1877 in the lungs of a tiger, was described by Kerbert in 1878. Nakagawa, Yokogawa, and others elucidated the life cycle. Several species have been described, differing but little in their morphology, and some authors believe that there is only one species parasitizing man, *P. westermani.*[142]

Characteristics and life cycle. The adult lung fluke is an ovoid reddish worm, 7 to 12 mm. long by 3 to 6 mm. in diameter, covered with a transparent cuticle which is studded with spines. The anatomy of a flattened specimen is shown in Figure 241. Two muscular suckers are present, one on the ventral surface, the *acetabulum,* the other at the anterior end, the *anterior sucker,* perforated by the mouth. The mouth opens into a muscular pharynx, followed by a thin esophagus. This divides into two blind *intestinal ceca* extending down the sides of the body. The nervous system is simple, and no special sense organs are present. The excretory system consists of a bladder opening at the posterior end and receiving collecting tubules which extend throughout the body, terminating in characteristic "flame cells." The circulatory system is rudimentary, consisting of indefinite channels through the parenchyma.

The adult fluke is hermaphroditic, both male and female reproductive systems occurring in the same individual. These systems are typically elaborate and, as in most flukes, are the most prominent structures of the body. In the female system a single ovary connects via an oviduct with the *oötype,* where the eggs are formed and receive their shells. In its course the oviduct receives the common *vitelline duct,* into which shell material comes from the branched *vitellaria,* glands occupying the sides of the dorsal surface of the body. The oviduct also has a diverticulum, the *seminal receptacle,* in which sperms are stored. An inconspicuous duct of unknown function, *Laurer's canal,* runs from the oviduct to an opening on the dorsal surface of the body. The oötype, surrounded by gland cells, *Mehlis' gland,* opens into the uterus, a long coiled tube which carries the completed eggs to the common genital pore near the acetabulum.

The male reproductive system consists of paired testes in the posterior part of the body, connecting by vasa efferentia with the vas deferens, which empties into the common genital pore along with the uterus. Part of the vas deferens is widened into a sperm reservoir, the seminal vesicle, and a more distal portion is the glandular *prostatic region.* The terminal portion forms a muscular copulatory organ, the *cirrus.*

The eggs produced by adult worms in the lung are coughed up and either escape in the sputum or are swallowed and passed in the feces. They are ovoid, averaging 60 by 90 μ in size, and show a removable cap, the *operculum,* at one end. The embryos are undeveloped at the time of escape from the host. They develop in from 2 to 6 weeks in water, where the eggs hatch by the opening of the operculum to release a free-swimming ciliated larva, the *miracidium.* This larva survives only a few hours unless it succeeds in penetrating the tis-

sues of a suitable intermediate host. Various snails of the genera Melania and Pomatiopsis can serve as hosts. In the lymph spaces of the snail the miracidium becomes an irregular, thin-walled sac, the *sporocyst*, growing to a final length of about 0.4 mm. Cell masses within the sporocyst enlarge, developing in about 1 month into twelve or more *rediae*. These escape by rupture of the sporocyst and grow to a length of about 0.3 mm. They differ from the sporocyst principally in having a rudimentary digestive tract and a *birth pore*. By a reproductive process similar to that in the sporocyst the first generation redia produces twelve or more second generation rediae. These escape by way of the birth pore and grow to a length of about 0.5 mm. Within each second generation redia arise twenty or more tailed larvae, or *cercariae*. The cercaria is essentially a rudimentary adult worm. It differs principally in having a small tail and two types of penetration organs, a stylet in the mouth region and several gland cells, opening at the anterior end, which secrete histolytic enzymes. About 3 months after invasion of the snail by miracidia the cercariae escape and move about in the water, dying in 1 or 2 days unless they find a suitable second intermediate host, a crayfish or crab of any of various genera, especially Astacus and Potamon.

The cercaria penetrates the softer part of the integument of a crab, loses its tail and grows in the tissues into the *metacercaria*, a near-spherical body about 0.4 mm. in diameter enclosed in a cyst wall. The full development requires about 1 month, after which the metacercaria is infective when eaten by man. In the small intestine the cyst wall softens, releasing the metacercaria, which penetrates the wall of the small intestine and reaches the abdominal cavity within a few hours. It wanders rather aimlessly but usually passes through the diaphragm within a few days and invades the lung, where it becomes encapsulated by the tissues and grows to the adult stage. Considering this apparently random migration it is interesting to note that the pleural cysts commonly contain two or three adults. After about 6 weeks eggs may be found in the sputum.

Paragonimiasis. The adult worms in the lung, and particularly the eggs they produce, cause tissue destruction, inflammation, and hemorrhage. Local pneumonic processes with cough and bloody sputum are characteristic. As in most worm infections, the damage is roughly proportional to the number of organisms present. In severe cases weakness and even death may result from the extensive lung injury. Aberrant worms in other tissues, such as the brain, may produce local injury.

There is no evidence concerning acquired *immunity* to Paragonimus. Human infections have been shown to persist at least six years in the absence of re-exposure, but it is said that they often clear up after several years.

Diagnosis of lung fluke infection depends on the finding and identification of characteristic eggs in the sputum or feces. As in other trematode infections, the size and structure of the eggs are characteristic.[86] Other trematode eggs may occur in the feces, but only those of Paragonimus normally occur in the sputum.

Epidemiology and control. Various mammals are susceptible to Paragonimus infection. Cats, dogs, mink, muskrats, and man are the usual natural hosts. Infection in lower animals is known in Asia, Africa, North and South America, including the United States. Human infection is limited by food habits, occurring commonly only in the Far East. The metacercariae in infected crustacea are destroyed only by thorough cooking or considerable exposure to pickling sauces, and in the endemic areas fresh water crabs, raw or slightly pickled, are considered a delicacy. It should be emphasized that the asexual reproduction in snails is obligate and that only the metacercariae and possibly the cercariae are infective for mammals. Thus, direct transfer from mammal to mammal does not occur, and numerical increase of an individual infection is unknown in the absence of re-exposure.

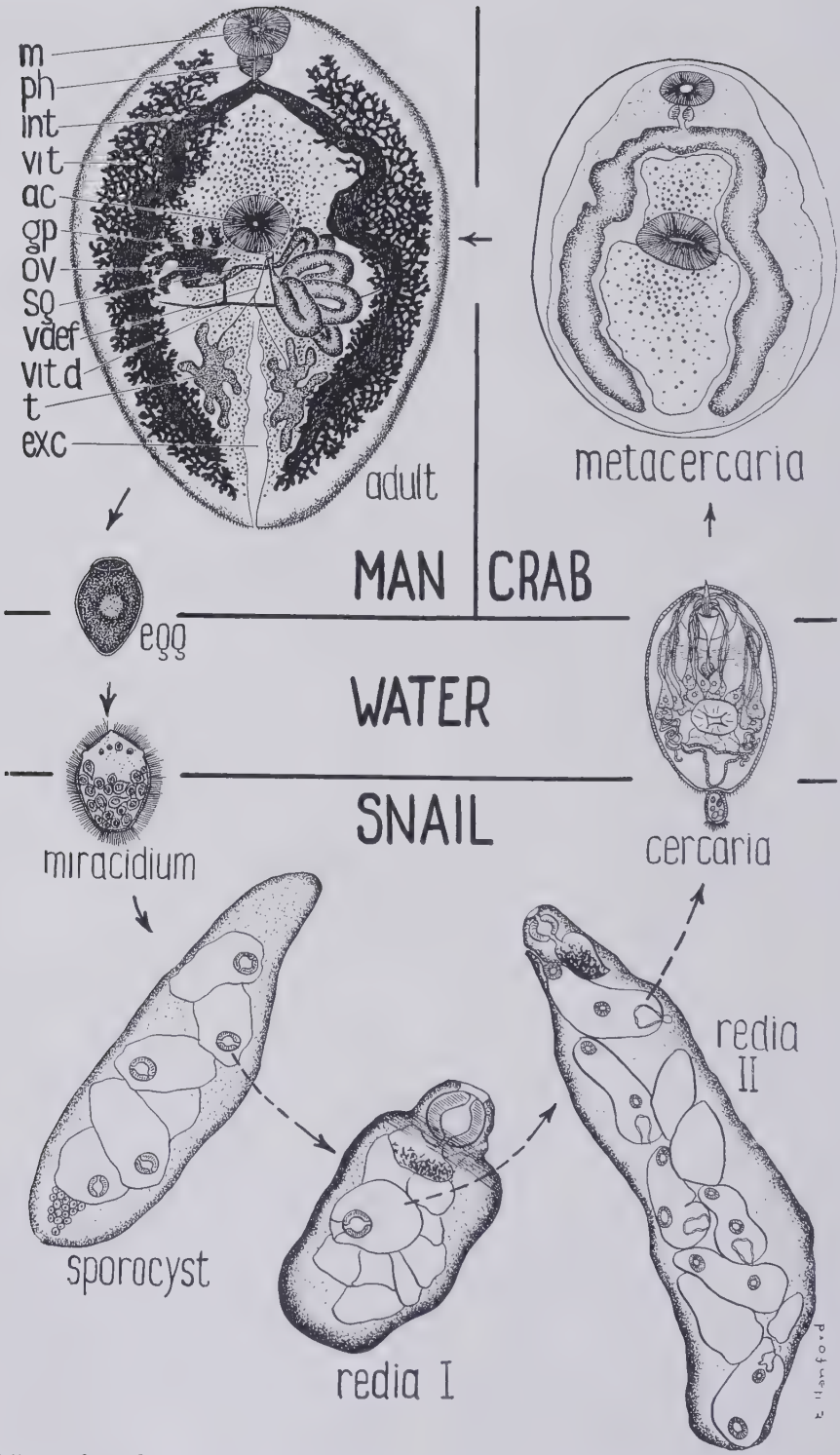


Figure 241. Life cycle and morphology of Paragonimus (larval stages redrawn from Ameel, 1934, × 120; adult worm, × 7): *m*, mouth; *ph*, pharynx; *int*, intestinal cecum; *vit*, vitellaria; *ac*, acetabulum; *gp*, genital pore; *ov*, ovary; *sg*, oötype; *vdef*, vas deferens; *vitd*, vitelline duct; *t*, testis; *exc*, excretory bladder.

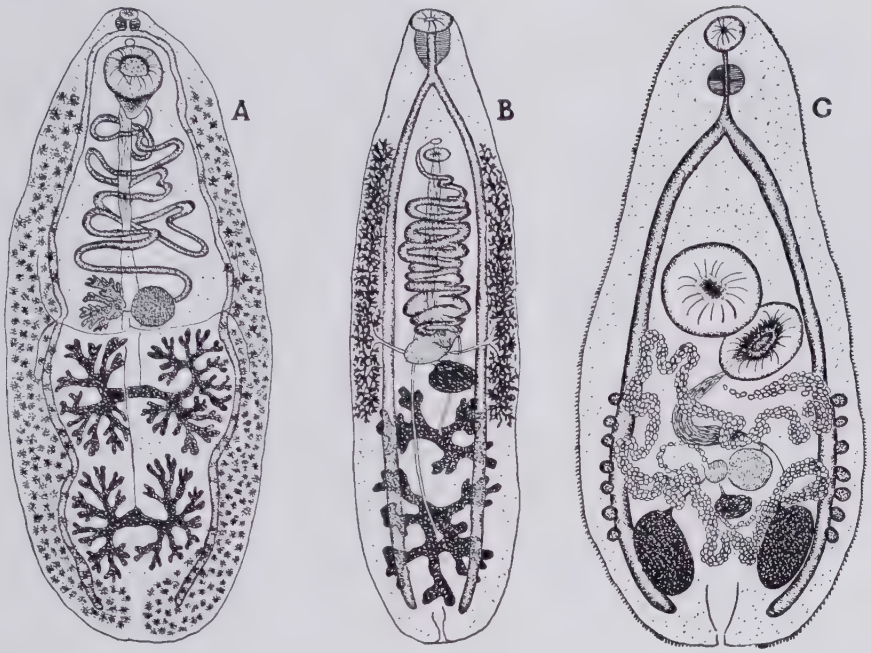


Figure 242. Trematode parasites of man. Note differences in magnification. *A, Fasciolopsis buski;* × 3. *B, Clonorchis sinensis;* × 5. *C, Heterophyes heterophyes;* × 60.

Chloroquine, in addition to its antimalarial activity, has been utilized with some success in a number of helminth infections including paragonimiasis. It is beneficial in early pulmonary infection but of dubious value in long-established infection or in ectopic cerebral parasitism. Good clinical results have also been reported with Bithionol.[141]

Chemotherapy of human infections is useless for control both because of its unreliability and because lower animals are an important reservoir. Destruction of snails has generally failed. The only effective control measure known is education to the dangers of eating improperly cooked fresh-water crustacea. It should be noted here that while such modification of food habits is the best control method for all human trematode infections except the blood flukes, established habits and prejudices often interpose difficulties in its execution. Yokogawa et al.[142] have recently reviewed the status of knowledge of paragonimiasis.

Fasciolopsis Buski. The large adults of this species attain a length of 7 cm. They are found attached to the mucosa of the small intestine of man and the pig. The infection is most common in China but occurs in other parts of Asia. The large eggs (average 140 by 80 μ) escape in the feces and develop in water, where the miracidia invade snails of the genera Segmentina and Planorbis. The development in the snail, like that of Paragonimus, comprises a sporocyst generation and two generations of rediae. The long-tailed cercariae encyst as metacercariae on aquatic plants, particularly the water chestnuts. These plants, eaten raw or peeled with the teeth, convey the infection to man or the pig. Symptoms are generally related to the intensity of infection. They consist of intestinal disturbances and generalized edema. The latter has been attributed to toxemia but may be merely nutritional, resulting from the food-robbing of the host by the worms. Severe infections may cause death. As in many other intestinal worm infections, hexylresorcinol is therapeutically efficient. Control is achieved by treatment of human infections, proper sewage disposal, and education to the dangers of eating raw plants from contaminated

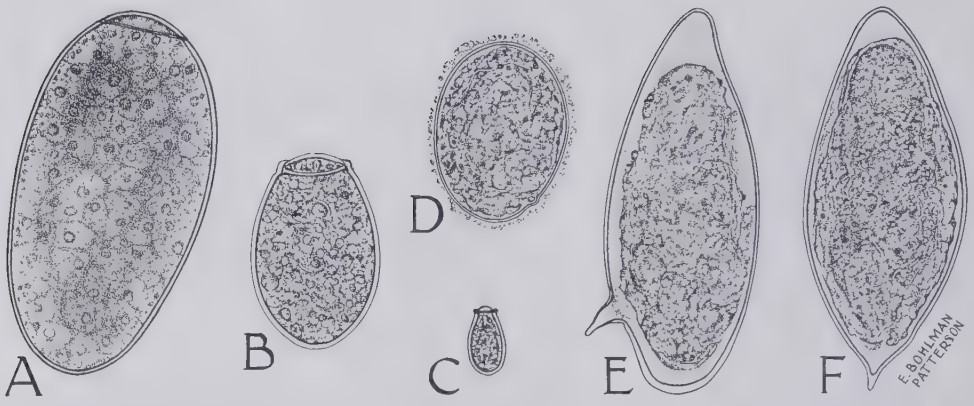


Figure 243. Trematode eggs in optical section. Camera lucida. Approx. × 310. *A, Fasciolopsis buski. B, Paragonimus westermani. C, Clonorchis sinensis. D, Schistosoma japonicum* (note that the shell has the halo of debris usually seen when this egg is found). *E, Schistosoma mansoni. F, Schistosoma haematobium.*

water. The problem is complicated by the practice, common in endemic areas, of using human feces as fertilizer.

The closely related *Fasciola hepatica* infects the livers of sheep and cattle, causing "liver rot," and is of great economic importance. It is important also because it was the first trematode whose life cycle was described. Occasional human cases are known, usually contracted by eating fresh watercress to which metacercarial cysts are attached. Human infection is relatively frequent and clinically important in Cuba, Chile, southern France, and other scattered areas where sheep or cattle husbandry accompanies the raising of leafy vegetable crops commonly eaten uncooked. *Fasciola gigantica* and *Fascioloides magna* of herbivores also occasionally infect man. Another economically important parasite of the liver of herbivores, *Dicrocoelium dendriticum,* has been found rarely in man.

Heterophyes Heterophyes. *Heterophyes heterophyes* is a small trematode, about 1.5 mm. long, of the intestine of man, cats, dogs, and other fish-eating mammals in Egypt, Asia Minor, and Asia. The eggs are small, averaging 25 by 16 μ. Metacercariae are ingested by man in insufficiently cooked or salted fish. The adult trematodes produce only minor gastrointestinal disturbances, but it is reported that the eggs are often deposited deep in the mucosa, whence they reach the general circulation and are localized in various distant tissues. The inflammation in these tissues, particularly the myocardium and brain, is said occasionally to cause serious symptoms or death.[33] Several related species attack man. The most important of these is *Metagonimus yokogawai,* which occurs in man and other fish-eating animals in the Balkans, Palestine, and Asia. A number of heterophyid flukes whose eggs cause extensive ectopic lesions have also been reported from man in the Philippines.[2]

Other Intestinal Trematodes. Several species of Echinostoma, Echinochasmus, and Euparyphium occasionally parasitize man. They are elongate trematodes with a ring of large spines on the head region. The human infection is acquired by ingestion of raw snails or mussels containing the metacercariae. Various mammals and birds are the normal hosts of the adult worms. Occasional human infections are also reported with *Gastrodiscus hominis* and *Watsonius watsoni.* Their life cycles are unknown, but since they chiefly parasitize herbivorous mammals it is presumed that the metacercariae are found on plants.

Clonorchis Sinensis. The Chinese liver fluke, *Clonorchis sinensis,* is a thin, elongated trematode, 1 to 2 cm. in length. The adult worm inhabits the smaller bile ducts of man, cats, dogs,

and other fish-eating mammals in China and Japan. The eggs, averaging 29 by 17 μ, pass out with the bile and escape in the feces. They are fully developed but do not hatch in the water. Upon ingestion by suitable snails, usually of the genera Parafossarulus and Bithynia, they hatch, and the miracidia become sporocysts in the lymph spaces. Rediae produced in the sporocyst give rise to long-tailed cercariae. These encyst as metacercariae in the tissues and on the skin and scales of various fresh-water fish. Metacercariae ingested by man hatch in the small intestine, and the young worms migrate up the bile ducts to develop into adults 3 to 4 weeks later. In rabbits experimentally infected, the young worms complete migration to the liver in hosts whose bile ducts have been ligated. Consequently, passage through the circulatory system to the liver or direct migration through the intestinal wall to the peritoneal cavity and into the liver surface may also occur.[140] Thorough cooking (100° C. for 15 minutes) is necessary to destroy the metacercariae. Infection of man occurs by ingestion of the metacercariae, possibly in drinking water or by contamination of the fingers in handling infected fish, but usually by consumption of insufficiently pickled or cooked fish, a favorite article of diet in the endemic regions. Hazards are increased in these areas by the practice of propagating fish for market in ponds fertilized with human feces.

Human infection is characterized by proliferation of the bile-duct epithelium and of the surrounding connective tissue. This results in liver cirrhosis, destroying liver parenchyma and obstructing portal blood flow. Intestinal disturbances, liver enlargement, and ascites are the common symptoms, severe infections resulting in death. No reliable drugs are known. Control, as in the trematode infections discussed above, depends principally on education concerning avoidance of undercooked infected food, in this case fresh-water fish.

A closely related species, *Opisthorchis viverrini*, is of considerable importance in northern Thailand where it infects 25 per cent of the population.[112] *Opisthorchis felineus* and *O. tenuicollis* infect fish-eating mammals and, less commonly, man in parts of Central Europe and Asia.

Human Blood Flukes: The Schistosomes. The high incidence of infection of man with the several species of schistosomes as well as the chronic debilitating disease produced places these organisms among the world's most important infectious agents.[106] The world incidence of infection is estimated to be greater than 4 per cent and some areas of endemicity are increasing in size.[89] Control measures and treatment of the individual are still inadequate. Bilharz, in 1852, first described trematodes in the veins of an Egyptian and subsequently infection with the schistosomes was termed "bilharziasis" in much of the medical literature. The worms are usually found in pairs in the portal venous system or in veins of the vesicle plexus with the female held in the ventrally grooved male gynecophoral canal. The split body of the male suggests the generic name for the group and unlike the other trematodes mentioned all blood flukes are dioecious. The adults attach to the walls of the blood vessels with their suckers and migrate to the smaller venules in copula for egg deposition. Occasionally they may develop in ectopic sites, probably being carried there by the circulation prior to attaining maturity. The blood flukes rely on a supply of carbohydrate and utilize glucose from the blood.[19, 135] In addition they ingest erythrocytes and have a globin-splitting enzyme.[126] *Schistosoma mansoni* is used here as an example of the group.

Morphology and life cycle. The paired adults of *S. mansoni* are normally found in the smaller mesenteric veins. The cylindrical female is 1 to 1.5 cm. in length by about 0.25 mm. in diameter. An anterior sucker surrounds the mouth and shortly behind it is a stalked acetabulum. The intestinal ceca unite before the middle of the body to form a single tube continuing to the posterior end. An oval ovary lies immediately anterior to the union of the intestinal ceca,

and the uterus, containing eggs, extends forward to open behind the acetabulum. The posterior half of the body is occupied by vitellaria. The male is shorter than the female, about 1 cm. long by about 1 mm. in diameter, its integument covered with coarse tubercles. The suckers and alimentary tract are similar to those of the female. The reproductive system consists of a cluster of eight or nine round testes in the anterior region emptying into a seminal vesicle, which opens to the ouside just behind the ventral sucker.

S. mansoni has large eggs, averaging 150 by 65 μ, with no operculum but with a prominent spine at the side. They are deposited in the fine venules of the mesenteric veins in the wall of the intestine, where they lodge after the retreat of the ovipositing worms and the collapse of the vessels which had been distended by the worm body. The lateral spine aids in fixing the egg in the vessel and by a combination of events, probably including secretion of lytic substances by the developing embryo, the egg passes through the vessel wall. From the tissues the eggs continue migration through the mucosa and into the lumen of the intestine. They are mature at the time they leave the body in the feces. On dilution of the feces with water, the shell ruptures, releasing the miracidium. In suitable snails, Biomphalaria in Africa and *Australorbis glabratus* in the Western Hemisphere, the miracidium penetrates and two generations of sporocysts are produced resulting, after about 6 weeks, in the production of cercariae with long, forked tails. Escaping into the water the cercariae swim actively or rest at the surface, dying in about 3 days if they do not reach a susceptible final host. Coming in contact with human skin they attach and actively penetrate the skin. The epidermis is passed quickly and the cercariae shed their tails and may reach lymph vessels in the dermis within 15 minutes or less. This rapid invasion of the unbroken host skin is accomplished through use of cercarial enzymes as well as by muscular activity. A major feature of cercarial morphology is the possession of sets of large unicellular "penetration glands" which are the source of mucoid secretions, collagenase-like proteases,

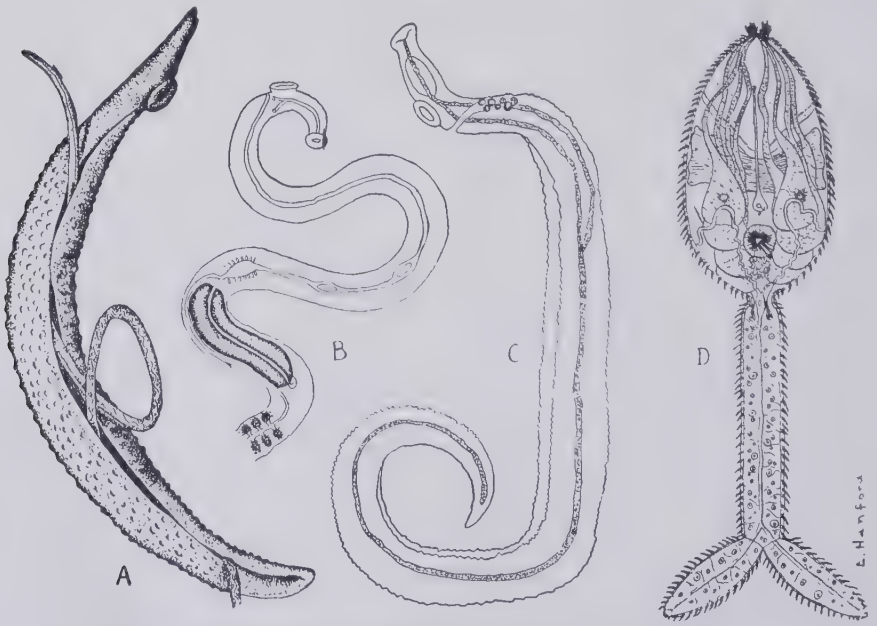


Figure 244. *Schistosoma mansoni.* Note differences in magnification. *A*, adult worms; × 8. *B*, anterior end of female worm; × 20. *C*, adult male; × 10. *D*, cercaria; × 180. (*A* and *C* redrawn from Manson-Bahr. *D* redrawn from Cort.)

and mucopolysaccharidases, all presumed to be of importance in invasiveness of these parasites.[80, 121] The parasite is carried via lymph and blood to the portal veins in the liver where maturation occurs and the adult forms mate and migrate distally in the portal system to the mesenteric veins. Four to 8 weeks after infection the sexually mature worms begin egg production. The infection may persist for over 25 years without re-exposure.

The other species of human blood flukes differ from S. *mansoni* in size, details of morphology, egg structure, location in the definitive hosts, types of snail hosts, and geographical distribution. S. *haematobium* adults occur predominantly in the venules of the urinary bladder wall, the eggs escaping in the urine. The eggs are large, 150 by 60 μ, with a terminal spine. The male, 1 to 1.5 cm. long, is covered with fine tuberculations and has four large testes. The female is about 2 cm. long. Snails of the genera Physopsis and Bulinus serve as intermediate hosts.

S. *japonicum* occurs in the venules of the intestine. The eggs average 80 by 65 μ, with a small lateral knob rather than a spine. They escape in the feces, the miracidium invading snails of the genus Oncomelania. The male is 1 to 2 cm. long, with a smooth integument, and has seven or eight testes. The female is 1 to 2.5 cm. long.

Schistosomiasis. The disease produced by the schistosomes varies with the infecting species, the number of adult worms present, and with the duration of infection. The pathology may conveniently be divided into the incubation period, from skin penetration to egg deposition, the acute stage, and the chronic stage. At the inception of the incubation period, skin penetration may cause a mild transient dermatitis. In the forms normally parasitizing man this is less severe than that produced in man by the penetration of cercariae normally parasitizing other vertebrates (see below). The larvae in passing through the lung may cause an eosinophilic infiltration and while developing in the portal system, parasite metabolic products cause a toxic hepatitis often accompanied by irregularly fluctuating fever and allergic manifestation such as urticaria. This is followed by the acute stage in which eggs are actively deposited in the tissues and are extruded into the lumina of the intestine or bladder. During this period trauma may produce intestinal hemorrhage and pseudodysentery in S. *mansoni* and S. *japonicum* infections. Hematuria is characteristic of this stage of S. *haematobium* infection.[51, 100] Even in these early stages numerous eggs may escape into the circulation rather than migrating through tissues to organ lumina. These embolic eggs lodge in numbers in the liver where they become enclosed in small granulomata (pseudotubercles) and in S. *haematobium* and S. *japonicum*, numbers commonly pass to the lung. In many instances in S. *japonicum*, relatively large numbers reach the brain, producing the symptomatology of cerebral damage. The chronic stage is due to the host's reaction to continuous egg-produced tissue damage. In S. *mansoni* the wall of the large intestine becomes thickened with scar tissue and rectal polyps of connective tissue with eggs may be extensive. Similarly, impairment of the small intestine and thickening occur with S. *japonicum*, and in both, cirrhosis of the liver is a most important sequel. With S. *haematobium* the wall of the urinary bladder is greatly thickened with fibrous connective tissue and the urethral lumen is commonly reduced.[63] The external genitalia are also commonly involved. Carcinomas of the rectum, liver and urinary bladder are often observed and in some areas a high correlation between these malignancies and schistosomiasis is claimed.

Concomitant with the liver damage found in S. *mansoni* and S. *japonicum* infections, circulatory impairment produces portal hypertension, splenic enlargement, esophageal varices and ascites.[6, 48] As briefly summarized above, the pathology produced varies with time and also with the differing site of egg deposition by the different species of

adult worm. Another important species difference in the disease is the fact that the female *S. japonicum* produces ten times the number of eggs per day as the *S. mansoni* female. Egg production of the female *S. haematobium* is believed to be intermediate between these.

Immunity. Direct evidence of functional immunity to schistosomes in man is lacking. Children and young adults commonly have severe infections which may terminate fatally. Some infected adults are found without symptoms of progressive disease under conditions where it may be assumed that they have periodic exposure to reinfection with *S. japonicum* or *S. mansoni.* Animals cured of initial infection show some resistance to reinfection and in monkeys, some individuals surviving initial infection are strongly immune to massive reinfection.[132] Recent evidence indicates promise that immunization by vaccination may be possible as rhesus monkeys inoculated with a nonhuman (Formosan) strain of *S. japonicum* exhibited considerable resistance to challenge with the human strain (Japanese).[66] In addition, mice inoculated with cercariae of *S. mansoni* that had been exposed to ^{60}cobalt radiation developed a relative immunity to reinfection with nonirradiated cercariae.[131]

A wide variety of serologic evidence of antibody production in schistosome infection exists and in individual human sera circumoval precipitins, miracidial agglutinins, cercarial agglutinins, complement-fixing, and other antibodies can be demonstrated.[74] In addition, serum proteins inhibiting cercarial penetration enzymes are also produced.[80] To date these serological phenomena cannot be correlated with the degree of functional or protective immunity of the individual.

Diagnosis. Laboratory diagnosis is based on the finding of characteristic eggs in the feces, urine, rectal biopsy, or rarely, liver biopsy. In very early or in late chronic stages the eggs may not readily be found and multiple examinations plus concentration techniques must be routine for diagnosis of individual infections. Immunologic techniques are also valuable both for the individual diagnosis and for epidemiological surveys. These include the production of precipitate about viable or lyophilized eggs (circumoval precipitin reaction), high titer hemagglutination of erythrocytes sensitized with adult antigens, intradermal reaction to adult antigens, binding of fluorescent antibody by preserved cercariae,[114] and a variety of other methods including complement-fixation and gel precipitation techniques.[74] Some of the reactions are group specific, persist after cure, or are negative in a high percentage of the lower age group. The circumoval, fluorescent antibody, and intradermal tests are of practical value if their limitations are understood.

Therapy. The most effective treatment of all three species is the intravenous administration of potassium or sodium antimony tartrate which properly administered will provide a high percentage of cures (84 per cent in *S. japonicum*).[17, 18] This tartar emetic requires slow administration on alternate days over a one-month period, is painful, and not practical for mass treatment. Consequently Fuadin (Stibophen), an intramuscularly administered antimonial, is a favored drug in many of the endemic areas. Characteristically, it sterilizes temporarily the female schistosome causing cessation of egg-laying and of symptoms referable to this damage. Properly used, cures may be effected. However, the cure rate is low since a proper course of therapy is seldom followed and the female worms are believed to recover. The mechanism of action of the trivalent antimonials is an interference with one of the enzymes (phosphofructokinase) of the helminth carbohydrate metabolism.[19] Miracil D, an orally administered drug, has been shown to be of some value in the treatment of *S. haematobium* infections and is of particular value in small children.

Epidemiology and control. The chief means of infection is by contact of the skin with water containing the cercariae.

This occurs especially in the working of rice fields fertilized with human feces, but also in bathing and chance contact. Infection may be acquired from drinking water and rarely as an intra-uterine infection. Man is the only important reservoir of S. *mansoni* and S. *haematobium* infection although both occur naturally in monkeys and rodent infections of S. *mansoni* have been described. The disease is for these two species perpetuated by the promiscuous discharge of urine and feces by man or their deliberate use as fertilizer in the cultivation of rice, sugar cane and similar crops. Infection of streams and irrigation canals used for ablution and the washing of clothes is important in maintenance of the infection. In contrast to these forms, S. *japonicum* has numerous reservoir hosts among domestic and wild mammals which pass eggs in feces and may maintain the infection in man's absence. S. *mansoni* is widely distributed throughout Africa wherever suitable snail intermediate hosts are found. The delta of the Nile is an endemic area of primary importance; it is common throughout tropical Africa to South Africa and in much of its range coexists with S. *haematobium*. Importation of S. *mansoni* with slaves established the disease in Brazil, Venezuela, Surinam, and in the West Indies including Puerto Rico. In these areas it remains of major importance and world incidence approaches 40 million. The present distribution of S. *haematobium* not only includes all of Africa suitable for its development but also parts of Arabia, Palestine, Lebanon, Syria, and small endemic areas in Portugal, and India. S. *japonicum* is limited to the Far East with important endemic areas in the Philippines, China, the Celebes, and Japan. A small focus is present in southern Thailand and a strain primarily parasitizing dogs and rats is found in Taiwan.

Control of schistosomiasis depends on destruction of the snail intermediate hosts, prevention of access of eggs to water containing snails, or avoiding contact with cercaria-containing waters. Some limited success has been obtained in snail control using copper sulphate or sodium pentachlorophenate as molluscicides.[89] Periodic drying of irrigation ditches is of some value for the control of snails transmitting S. *mansoni* and S. *haematobium* but of little value in S. *japonicum* control as the snail host is operculate and resists desiccation. As it does not survive well in ponds or in well-drained areas, ponding and drainage are utilized in control. In limited areas having special characteristics the introduction of a competitor, the snail Marisa, has eliminated Australorbis, the host of S. *mansoni*.[103] Safe sewage disposal and increased sanitation has not met with outstanding success as a control measure since the customs of the people as well as the economics of the endemic areas operate against these methods. Avoidance of contact with infected water is difficult in some areas since it is also associated with religious habits of ablution; in other areas only infected waters are available for drinking and general cleansing, and it has not been economically possible to provide safe water supplies.

Other schistosome species. A number of species commonly parasitizing ungulates of Africa and India, including S. *bovis,* and S. *spindale,* have been reported as occurring in man. Several hundred cases of S. *intercalatum* in man have been reported from tropical Africa. This form has a terminal-spined egg slightly larger than that of S. *haematobium*. The eggs are passed in the feces rather than in the urine.

Schistosome dermatitis. Several species of schistosomes, especially in the genus Trichobilharzia, may invade the skin of man to cause severe allergic dermatitis. These worms, which normally parasitize birds or lower mammals, apparently cannot mature in man. While dermatitis-producing schistosomes have been recorded from scattered areas throughout the world, excepting Africa, they are prominent only in parts of Europe and North America. Certain lakes in the north central states, particularly in Michigan and Wisconsin,

were formerly notorious for the "swimmer's itch" contracted by bathers.[30] Snail destruction with copper salts has successfully controlled the disease. The dermatitis can be treated with antiallergic ointments. One of the parasites producing schistosome dermatitis, *Schistosomatium douthitti,* is of especial interest because it is easily studied in laboratory mice. Recently, avian schistosomes causing a marine dermatitis have been described from salt water beaches of Hawaii, California, and the eastern United States.[26]

Cestoda

The cestoda, or tapeworms, generally consist of a colony-like chain of flattened segments, each of which is a semi-independent unit with a complete set of reproductive organs. A *scolex* at the anterior end of the chain serves to anchor the worm in place, and the nervous and excretory systems are shared by the whole organism. There is no alimentary tract, soluble food substances being absorbed through the body surface. All tapeworms are parasitic, the adults usually in the alimentary tract of the definitive host and the larval stages in the tissues of an intermediate host.[137] Two orders of tapeworms, the Pseudophyllidea and Cyclophyllidea, contain human parasites. The Pseudophyllidea are discussed later (see *Diphyllobothrium latum*). Most of the tapeworms of man belong to the Cyclophyllidea, of which *Taenia solium* will serve as an example.

Taenia Solium. The pork tapeworm, *Taenia solium,* was known in ancient times. Küchenmeister, in 1855, first suspected its relationship to "bladderworms" in pork and demonstrated their transformation into the adult worms in the intestine of a condemned criminal.

Characteristics and life cycle. In the intestine of man the adult *T. solium* attains a length of 2 to 3 meters. Buried in the intestinal mucosa of the host is the scolex, or "head," a rounded cubical organ about 1 mm. in diameter bearing four large, cup-like suckers and a pad, the rostellum, with twenty to thirty-five hooks. Behind the scolex is a thin neck which merges into a region of proliferation of segments, or *proglottids.* As new segments are produced, the older ones are pushed back, developing sexually, until near the middle of the worm mature proglottids are found. They average 0.5 cm. square. The genital pore occurs on either side of the proglottid, alternating irregularly from segment to segment. The male reproductive system consists of small follicular testes scattered throughout the dorsal part of the segment, emptying through vasa efferentia into a coiled vas deferens which ends in the muscular copulatory organ, or cirrus, at the genital pore. In the female system the vagina extends from the genital pore to the oötype in the posterior central part of the segment. On either side of the oötype are ovaries and behind it the vitellaria. A blind uterus extends forward from the oötype.

As the segments move toward the posterior end of the worm, the uterus fills with eggs, becoming branched to fill most of the segment in the *gravid* proglottid, which is about 0.5 by 1 cm. in size. In *T. solium* there are about ten branches on each side of the uterus. The gravid segments become detached singly or in small groups and pass out with the feces. Before or after escape from the body they disrupt, releasing large numbers of eggs. These eggs, averaging 35 μ in diameter, are nearly spherical and provided with a thick "shell" of characteristic porous structure. Within the shell is seen the *onchosphere,* an embryo bearing six hooks.

Ingested by a suitable intermediate host, the eggs hatch in the intestine and the embryos penetrate the intestinal wall to enter the blood or lymph. They are carried to various parts of the body, developing in the tissues in about 2 months to the infective stage. Infection is most intense in the muscles, but other tissues also contain the parasites. This stage, a *cysticercus,* consists of a scolex like that of the adult, a short neck, and a fluid-filled sac about 1 cm. in diam-

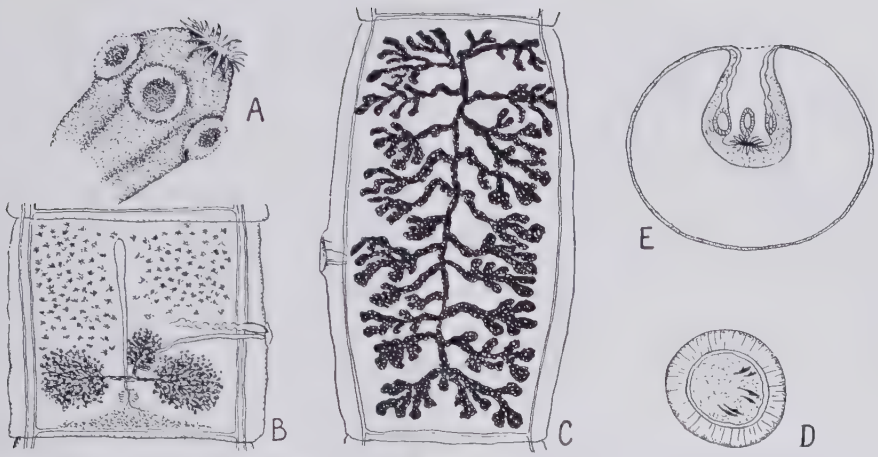


Figure 245. *Taenia solium.* *A*, scolex; × 20. *B*, mature proglottid; × 5. *C*, gravid proglottid; × 5. *D*, egg; × 500. *E*, cysticercus; × 5. (Adapted from various sources.)

eter. Because of the sac, this larval stage is commonly called a "bladderworm." In the tissues the scolex is invaginated into the bladder. In addition to the pig, other animals may harbor the cysticerci. Among them is man, in whom they occur in various tissues including the brain.

Man acquires the adult tapeworm by ingestion of undercooked pork containing the cysticerci. In the intestine the bladder is digested away, the scolex attaches to the intestinal wall, and the adult worm develops by growth from the neck region. By 2 months after infection gravid proglottids containing eggs are being passed in the feces. Infection may last for many years.

The human disease. Adult pork tapeworms usually occur singly in the intestine of man. They often cause no noticeable symptoms, though general intestinal discomfort may occur, and in children nervous disturbances are sometimes seen. The ravenous appetite popularly associated with tapeworm infections is actually uncommon, loss of appetite being more frequently observed. In contrast with infection by the adult worm, tissue infection with the cysticerci is dangerous. The larvae act like benign tumors; growth is slow and terminates naturally with full development. The injury results from pressure, and its seriousness, therefore, depends on the location of the larvae. In most muscles and connective tissues they are of no consequence, but larvae in the eye may affect vision. In the brain they may give rise to epilepsy or other manifestations of local pressure.

There is no evidence of acquired *immunity* to *T. solium* in man, though it has been suggested that the rarity of multiple infections in the intestine indicates immunity to superinfection. In individuals harboring the cysticerci, complement-fixing and precipitating antibodies are detectable. They react with extracts of other tapeworms as well as *T. solium.*

Laboratory *diagnosis* of intestinal infection with *T. solium* is based on recovery of eggs or gravid proglottids from the feces. The eggs are not distinguishable from those of the beef tapeworm (see below), but the gravid segments can be identified by the smaller number of uterine branches, about ten on each side as against more than fifteen in the beef tapeworm. Diagnosis of the cysticercus infection is rarely made before operation or necropsy, but the immunological reactions mentioned above have been used. Quinacrine hydrochloride (Atabrine) is used in treatment by tablet or sometimes by duodenal intubation. Extract of male fern (Oleoresin of Aspidium), an old therapeutic agent, is still used in

some instances. There is no treatment known for the larval infection except surgery.

Epidemiology and control. The larvae of *T. solium* occur most commonly in pigs but are found also in man, monkeys, sheep, camels, and dogs. Before 1850 in Berlin 2 per cent of human autopsies showed the cysticerci, but the incidence is now much lower. Pigs acquire the infection by contamination of their food or water with human feces. In some areas of the tropics and the Far East pigs, because of their coprophagic habits, are responsible for the relative cleanliness around small rural villages. Larval infections in man are incurred chiefly by contamination of food, water, or fingers with eggs from human feces, but it is also possible that in the intestine of an individual harboring the adult worm the eggs may hatch without reaching the outside. Whether or not eggs must leave the body before they are infective, a person with the adult worm in his intestine is a constant hazard to himself as well as to others. Man, the only known host for the adult tapeworm, acquires the infection by ingestion of undercooked pork, which often contains large numbers of larvae. Freezing and thorough cooking kill the larvae, but ordinary pickling and smoking are ineffective. Government meat inspection reveals most infected carcasses and has been the most effective single control measure. Other control methods are directed at the prevention of contact between pigs and human feces.

Taenia Saginata. The beef tapeworm, *Taenia saginata,* resembles *T. solium* in morphology and life cycle, but several differences are noteworthy. The scolex is "unarmed," lacking the hooked rostellum of *T. solium.* The adult worm is usually about 5 meters long, but occasionally is much longer. The gravid proglottids are distinguished from those of *T. solium* by a larger number of uterine branches, usually fifteen to twenty on each side. Man is the only known host of the adult. The cysticerci develop in cattle and other ruminants, human infection resulting chiefly from consumption of undercooked beef. Rare cases of human infection with the larvae have been claimed, but it is probable that they were abnormal cysticerci of *T. solium.*

Echinococcus Granulosus. The adult of *Echinococcus granulosus* inhabits the intestines of dogs and related species. It is a minute worm 0.25 to 0.5 cm. in length, consisting of an "armed" scolex and three proglottids, one immature, one mature, and one gravid. Large numbers of the adults may occur in the intestine of an infected dog.

The natural intermediate hosts are sheep, cattle, and other ruminants, but a wide variety of animals are susceptible, including man, in whom the larva causes a serious disease. This larva is markedly different from those of the beef and pork tapeworms discussed above. In the viscera of an infected animal it attains a diameter of about 1 cm. after 5 to 6 months. It is infective after 8 months or more but continues to grow, often, after several years, reaching a diameter of more than 20 cm. The larva, known as a "hydatid cyst," is a spherical, fluid-filled sac composed of a thick cuticular wall with a thin *germinative epithelium* on its inside surface. From this germinative epithelium are formed two types of structures. Buds may appear and grow into stalked vesicles, *brood capsules,* on the inner surface of which stalked cysticercus-like "scolices" are produced. These brood capsules may become detached as *daughter cysts,* enlarge, and produce brood capsules and scolices within themselves. In the large cysts there may thus be produced many thousand infective scolices, each of which can develop into an adult worm if the cyst is eaten by a dog feeding on the viscera of an infected animal. Thus *E. granulosus* differs from *T. solium* chiefly in the fact that multiplication occurs in the larval as well as the adult stage. The predominant site of larval infection is in the liver. The lung is next in importance, and cysts may occur also in practically every other organ.

Hydatid disease in man. Echinococcosis, or hydatid disease, is characterized

by two general types of manifestations. First, hypersensitivity develops to components of the hydatid fluid, and accidental rupture of a cyst may cause serious, even fatal, reactions. Second, and most important, the growing cyst acts like a tumor, the injury resulting from pressure effects and depending on the localization of the cyst. Rupture of a cyst may give rise to new cysts produced from scolices, daughter cysts, or fragments of germinative epithelium. *Osseous* cysts, occurring in bone, may weaken the bone by erosion of its structure.

There is no information available concerning acquired *immunity* to the larval infection in man, though in immunized sheep the cysts develop somewhat abnormally. Dogs injected with antigens derived from the larvae are immune to infection with the adult worm.

Laboratory diagnosis is usually based on immunological reactions. The precipitin test and the Casoni intradermal reaction are both useful but remain positive after removal of the cysts. The most reliable test is the complement-fixation reaction, utilizing merthiolate-preserved cyst fluid as an antigen.

Epidemiology and control. The normal life cycle of *E. granulosus* involves dogs and sheep or cattle, man being an accidental host of the larva. For this reason, the infection is most common in the great grazing regions of the world, particularly Australia and New Zealand, North and South Africa, Iceland, and southern South America. The incidence in cattle and sheep occasionally exceeds 50 per cent. Various wild mammals may also be involved, among them moose, reindeer and wolves in northern North America. Transmission of Echinococcus eggs to food by sarcophagic flies from dog feces has been experimentally demonstrated and may be presumed to occur naturally.

Control of the infection in domestic animals depends chiefly on preventing dogs from eating offal. Possibly infected waste material should be buried or burned. Prophylaxis of human infection requires measures to reduce the chance of contamination of human food and water by feces of dogs.

Echinococcus Multilocularis. The adult of *E. multilocularis* differs from *E. granulosus* only in fine morphological detail. The larva, however, develops differently from the discrete, spherical cyst produced by the latter, forming an *alveolar cyst* which spreads malignantly as a foaming mass of minute, budding globules, each containing, in the normal hosts, a few scolices. The liver is usually infected, and it is extensively destroyed by the exuberant growth of the larval tissue.[133]

E. multilocularis has been found in central Europe and the Aleutian Islands but probably also occurs in northern Asia. The adult hosts are foxes, and the larvae normally parasitize wild rodents. Sporadic larval infections occur in man, causing usually fatal destruction of liver tissue.

Alveolar hydatid was known in man for nearly a century before, in 1951, Rausch and Schiller recognized it as a distinct species. It had previously been considered an abnormal form of *E. granulosus*. The name *E. alveolaris* has also been applied to it.

Hymenolepis Nana. The human dwarf tapeworm, *Hymenolepis nana,* is a small worm, 1 to 4 cm. in length, with mature proglottids 0.5 to 1 mm. in breadth. The scolex is similar to that of *T. solium* but smaller, about 0.25 mm. in diameter. The mature proglottids are markedly different in appearance from those of *T. solium,* the width being more than four times the length. Three large testes occur in each segment. The uterus in the gravid segments is a large, irregular sac. The gravid segments are usually destroyed in the intestine, releasing the eggs, which are found in the feces.

H. nana is unique among human tapeworms in not requiring an intermediate host. Ingested eggs hatch in the intestine of man to release an embryo which invades the tissues of an intestinal villus. Here, in about 4 days, it develops into a *cysticercoid,* a larva with a small bladder and a solid tail. This larva breaks out of the intestinal wall, attaches to the

Figure 246. Nematode eggs and larvae. *A, Ascaris lumbridoides. B, Necator americanus. C, Enterobius vermicularis. D, Trichuris trichiura. E, Strongyloides stercoralis* (rhabditiform larva). × 400.

mucosa, and develops into the adult worm. Eggs are found in the feces about 2 weeks after infection. It is reported that several types of insects can serve as intermediate hosts, but they are probably unimportant in human spread of the parasite. Large numbers of worms are commonly found in an infected individual, and they may give rise to intestinal and nervous disturbances in children. Epileptiform convulsions may occur. Quinacrine hydrochloride (Atabrine) and extract of male fern are useful drugs for elimination of the worms.

H. nana occurs naturally in man, monkeys, and rodents. Although the form in rodents has been separated as a distinct species, *H. fraterna*, it is generally considered a variety of *H. nana*. The parasite is cosmopolitan. It is much the most common tapeworm of man in the United States, especially prevalent in children, but occurring in all age groups. Age resistance has not been shown in man but is experimentally demonstrable in rats and mice.

The lack of an intermediate host permits direct spread from man to man. It is not known whether the eggs can hatch without having reached the outside. However, an infected individual is particularly liable to reinfection from fingers contaminated with his own feces.

The closely related *H. diminuta* of rats and mice is transmitted by several species of insect intermediate hosts. Occasional human infections have been reported, mostly in children.

Tapeworms of Lower Animals. Species of the genus Multiceps exhibit the adult stage in dogs and larval stages in sheep. The larva, known as a *coenurus*, resembles a cysticercus but has a number of scolices attached to a single bladder. In the brains of sheep the larvae produce a fatal disease. Occasional cases have been reported in man.

Taenia pisiformis is a common tapeworm of dogs and cats, the larvae developing in the abdominal cavity of rabbits. *T. taeniaeformis* occurs commonly in the intestine of cats. The larvae, strobilocerci, normally develop in the liver of rats or mice. There is considerable evidence that in the rat the larvae produce malignant tumors. These have also been produced by larval extracts in the absence of the living parasite and once developed are metastasizing and transplantable.[35] This species is of interest for a number of immunological phenomena. Definite sex resistance is shown by the fact that female rats acquire fewer larvae from a given dose of eggs than do males. Castration of females or injection of male sex hormones reduces their natural resistance, while female hormones increase the resistance of the males.[23] Acquired immunity is dependent in part on antibodies transferable with serum. Two antibodies are detectable. One acting early in the infection prevents development of the larvae; it is elicited by both infection and vaccination and is removed from the serum by absorption with worm substance. The

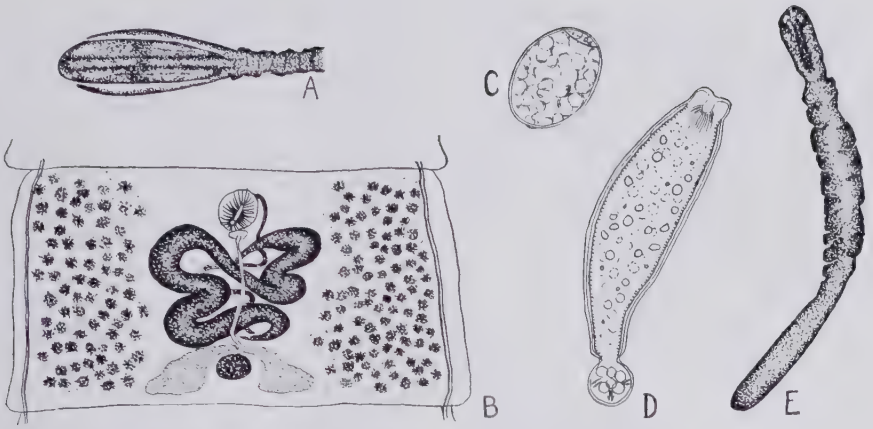


Figure 247. *Diphyllobothrium latum*. *A*, scolex; × 10. *B*, mature proglottid; × 6. *C*, egg; × 250. *D*, procercoid; × 100. *E*, sparganum; × 8.

other acts late in the infection, aborting the development of the larvae in the liver; it is elicited by infection but not by artificial immunization and is not absorbable with parasite material.

Dipylidium caninum, a common parasite of the intestines of dogs and cats, measures 10 to 50 cm. in length. Each elongate, rounded mature proglottid contains two sets of reproductive systems, one opening on each side of the segment. The uterus breaks up into pouches, each containing several eggs. Dog lice and various species of fleas serve as intermediate hosts, the cysticercoids in the insects being infective when ingested. Young children occasionally harbor the adult tapeworm.[93]

Diphyllobothrium Latum. The fish tapeworm *Diphyllobothrium latum* (or *Dibothriocephalus latus*) is the most important human parasite in the order Pseudophyllidea. It differs markedly in structure and life cycle from the species discussed above. It is a more primitive type, in many respects showing strong resemblances to the trematodes.

Morphology and life cycle. The adult worm in the intestine of man is very large, often exceeding 10 meters in length. The scolex is an elongate ovoid structure, about 1 by 2.5 mm. in dimensions, bearing, instead of the circular suckers of the cyclophyllidea, two elongated grooves which serve as attachment organs. The mature proglottid is broader than long, about 4 by 10 mm. in size. The small testes are scattered throughout the lateral dorsal regions, emptying by fine ducts into the vas deferens. This coiled tube runs from the posterior center of the segment forward to the muscular cirrus, which opens into the ventral genital pore in the middle of the anterior region. The vagina extends from the genital pore back to the large oötype in the center of the posterior region. The ovaries lie on either side of the oötype, and the vitellaria are small follicles scattered throughout the lateral ventral regions. Arising from the oötype, the egg-filled uterus coils throughout the center of the proglottid, ending at the uterine pore just behind the genital pore. This permits escape of the eggs without rupture of the proglottid, so that, contrary to the usual situation as seen in *T. solium*, the eggs are released into the intestinal contents and escape in the feces. The spent proglottids at the end of the worm break off and are passed in the feces.

The egg is undeveloped when it escapes in the feces, embryonation requiring a week or more in water. Like the eggs of most trematodes it is ovoid, with an operculum. Average measurements are 65 by 45 μ. Hatching occurs by opening of the operculum to release a free-swimming, spherical, onchosphere covered with long cilia. This embryo, the *coracidium*, dies within a few days

unless it is ingested by a suitable small crustacean, one of several species of copepods of the genera Cyclops and Diaptomus. Losing its cilia, the embryo reaches the body cavity of the copepod and becomes, in 2 to 3 weeks, an elongated solid body with a round tail bearing the six hooks seen in the embryo. This stage, the *procercoid,* is about 0.5 mm. long.

If the infected copepod is eaten by a fish, the procercoid penetrates the wall of the intestine and reaches the viscera or muscles. Here, in a week or more, it transforms into the *sparganum,* a wormlike stage 0.5 to 2 cm. long with a rudimentary scolex at the anterior end. This stage, ingested by man in undercooked fish, develops to the adult worm in the intestine, and eggs may be found in the feces after 3 weeks or more. If the infected fish is eaten by another fish, the spargana become established in the tissues of the second fish and remain infective for man. Human infections have been known to persist for years.

The human disease. *D. latum* commonly produces multiple infections, a number of worms occurring in the same intestine. The adult worms usually cause no symptoms, though in heavy infections intestinal disturbances may occur, sometimes with generalized edema. A small proportion of infected individuals show severe anemia, indistinguishable from pernicious anemia. It has been shown that the tapeworm concentrates vitamin B_{12} in its tissues, and it is clear that the consequent deprivation of a susceptible individual precipitates the anemia.[134] Satisfactory data on acquired immunity are not available. Laboratory diagnosis depends on identification of the eggs in fecal smears. As with the other tapeworms, quinacrine hydrochloride is the drug of choice.

Epidemiology and control. The adult worm parasitizes man, cats, dogs, bears, and other fish-eating mammals, but man is probably the principal source of infection in endemic areas. Susceptible copepods are widespread, and a variety of fishes can serve as second intermediate hosts. Endemic centers are known in northern and central Europe, northern Asia, Japan, parts of Africa, and the Great Lakes region of North America. Infection is especially widespread in Finland, where the incidence has been estimated at 20 to 25 per cent, and where most of the cases of tapeworm anemia have been reported. Personal protection depends on thorough cooking or freezing of fish from endemic areas and care in handling of such fish. Other control methods are aimed at reducing the pollution of bodies of water with untreated human feces and at the supervision of transportation of possibly infected fish.

Related species. Several related species of tapeworms, normally parasites of lower animals, are reported occasionally in man. Man also occasionally harbors the spargana of several species of Diphyllobothrium, normally parasites of the dog and other carnivores. *D. mansoni* is a common example.[94] Infection with these larvae in the subcutaneous tissues, muscles, or eye is known as sparganosis. Among the larvae infecting man is the very rare parasite, *Sparganum proliferum,* which multiplies enormously in the subcutaneous tissues and may cause death. The adult of this species is unknown. Larval infections with Diphyllobothrium usually result from ingestion of copepods in drinking water. In parts of the Orient split frogs are sometimes used as poultices, and human sparganosis has resulted from the migration of spargana from the frog tissues into the exposed tissues of man.

NEMATODA

All the important human parasites in the phylum Nemathelminthes (Aschelminthes of some authors) belong to the class Nematoda. They are cylindrical, elongated worms, unsegmented and with a body cavity which is not lined with a peritoneum of mesodermal origin as in higher animals. Symmetry is primitively bilateral. Male and female reproductive systems usually occur in

separate individuals noticeably different in general appearance.[25, 115] In some parasitic forms, parthenogenesis and protandry occur. Free-living nematodes are among the most numerous of animals and are found in a tremendous variety of habitat. Parasitic species in plants and animals are also abundant, and wild vertebrates are usually parasitized as are many invertebrates. Nematodes are important as disease agents of most domestic animals and the majority of mankind is infected with one or more species.

The structure of the nematodes parasitizing man varies greatly, and in size alone species occur which are too small to be readily seen with the unaided eye as well as forms over one meter in length. The life cycles are also varied from simple direct forms of development to those requiring several hosts. Among the simple forms, the pinworm of man, *Enterobius vermicularis,* will serve to illustrate the morphology and life history.

A word of caution is necessary here. The order of presentation of species in this section, based primarily on life cycles, cuts sharply across the lines of zoological classification, separating closely related types and bringing together types of very different structure.

Enterobius Vermicularis. The human pinworm, *Enterobius vermicularis,* normally inhabits the upper large intestine of man, occasionally invading the female genito-urinary system. The female worm, about 1 cm. long by 0.5 mm. in diameter, is a spindle-shaped organism with a long, pointed tail (see figure). The outer covering is a smooth, impervious, flexible cuticle, characteristic of nematodes in general. This cuticle, together with the fact that the body musculature is exclusively longitudinal, gives the worms a characteristic bending, wriggling type of movement. Among nematodes the cuticle shows great variety of external structures—knobs, fins, papillae, etc.—which are of value in technical identification of species. The alimentary tract consists of a mouth at the anterior end, a muscular esophagus with a posterior bulbous enlargement, and a thin-walled intestine emptying through the anus near the posterior end. The female reproductive system empties through a short vagina a little in front of the middle of the body. Extending forward and back from the vagina are two separate egg-producing systems (one to several in other nematodes). Each consists of a broad uterus filled with eggs, followed by a short narrow oviduct, which terminates in a long, thin ovary coiled about in the body cavity.

The male worm is smaller than the female, 2 to 5 mm. in length and 0.1 to 0.2 mm. in diameter, the posterior end coiled into a tight spiral and lacking the pin-like tail of the female. The male reproductive system is a single tube consisting of a testis, a vas deferens, a seminal vesicle, and a muscular ejaculatory duct, provided with glands, which empties with the intestine into a cloaca. A sharp copulatory spicule can be extruded through the anus. The posterior end has minute expansions (more prominent in many other nematodes) which form a clasping *bursa* used in copulation.

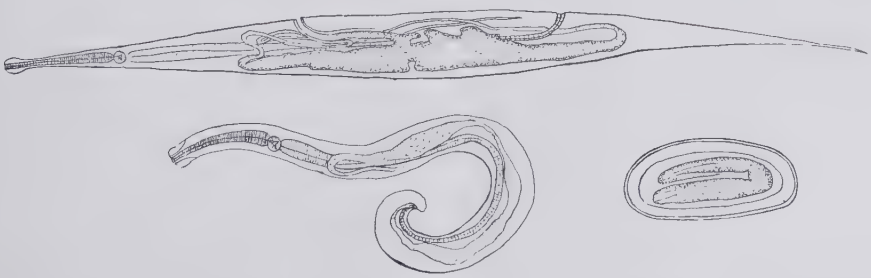


Figure 248. *Enterobius vermicularis. Above,* female, × 15. *Left,* adult male, × 15. *Right,* egg, × 300. (Redrawn from Faust after Leuckart.)

Some of the eggs escape in the intestine, passing out in the feces, but usually the gravid females migrate out of the anus to relase their eggs on the surrounding skin. The eggs average 55 by 25 μ in size, usually show mature embryos when passed, and are infective within a few hours. Ingested by man they hatch in the small intestine, where the worms mature and mate before passing down to the large intestine. Eggs appear in the feces about 2 weeks after infection. The adults in the intestine are short-lived, but infection is maintained or increased in the individual by self-infection. Hatching of eggs on the perianal surfaces with subsequent migration of larvae into the anus (described as "retroinfection") occurs in related species and it has been suggested this may also occur in man.

Pinworm infection in man. The adult worms in the intestine have little or no effect on the host. However, they occur in the vermiform appendix, and since they are said to be more common in diseased than in normal appendices, they have been suspected of an etiological role in appendicitis. During the nocturnal emergence of the female and with egg deposition in the perianal region itching occurs which may be intense, leading to scratching, scarification, weeping eczema, and bacterial infection. Worms may enter the female genitalia and cause similar irritation and occasionally migrate into the peritoneal cavity via the fallopian tubes. In children pruritus ani, sleeplessness, and resulting irritability are common. A slight eosinophilia may accompany infection and no immunity is known to develop.

Diagnosis is based on identification of eggs in the feces or from the perianal skin. The best method utilizes a strip of cellophane tape. The gummed side is pressed repeatedly on the perianal skin and then applied to a microscope slide for examination. Piperazine and pyrvinium chloride are therapeutically effective, but treatment is often followed by reinfection from the environment.

Epidemiology and control. Man is the only host of *E. vermicularis*. The eggs are resistant to drying and may contaminate clothing, bedding, and house dust, thus maintaining a constant source of infection in homes and institutions. The parasite is cosmopolitan. It is especially common in children, probably largely because their insanitary habits allow greater chances of spread. Treatment of cases is useful in control, provided sanitary measures are taken to minimize reinfection from the local environment.

Related species. Similar nematodes occur in many lower animals and are sometimes economically important as minor disease agents. *Syphacia obvelata,* a species found in rats and mice, is an occasional parasite of man and may readily be obtained for study from rodents. The eggs resemble those of *E. vermicularis* but are twice as large.

Trichuris Trichiura. *Trichuris trichiura* is a frequent inhabitant of the cecum, appendix, and upper colon of man. The basic morphology of *T. trichiura* is similar to that of *Trichinella spiralis* which is illustrated in an accompanying figure. The anterior three fifths of the adult worm, containing the nonmuscular esophagus, is atenuated, and the general appearance of the body has given rise to the common name "whipworm." Male and female worms are similar in size, 3 to 5 cm. long. The adult worms, embedded in the intestinal mucosa, have been accused of a role in the pathogenesis of appendicitis, but the evidence is inconclusive. Light infections are common throughout the population of the world's tropics and subtropics, are relatively without symptoms, and are unimportant to the infected individual. However heavy infections, particularly in children, cause a hyperemic mucosa of the colon and rectum, chronic diarrhea, extensive inflammation and rectal prolapse.[71] Consistently effective drugs were not available until very recently, when it was shown that dithiazinine, a cyanine dye, is highly efficient in eliminating the worms from the intestine. The eggs passed in the feces are thick-walled, with a plug at either end, 53 by 22 μ in size. They are very

resistant, surviving for months in contaminated soil. The embryos, undeveloped at the time of passage, require 2 weeks to several months, depending on temperature and humidity, to reach the infective stage. Ingested with contaminated food or water, they produce adult worms in the intestine. *T. trichiura* infection is world-wide but especially abundant in the tropics and subtropics because of poor sanitation and the effect of climate on development of the eggs.

Ascaris Lumbricoides. Widely known simply as the "roundworm" of man, *Ascaris lumbricoides* is a large parasite of the small intestine. The white or flesh-colored females measure 20 cm. or more in length by 5 mm. in diameter, the males, 16 cm. by 3 mm. The characteristic eggs, 45 to 75 by 35 to 50 μ, have a smooth inner and a roughly tuberculated outer shell. They are undeveloped when passed in the feces. In soil or water they become infective in nine days or more, depending on environmental conditions. They may survive for several years despite drying, bacterial contamination, or adverse chemical conditions. Ingested in food or water they hatch in the intestine. The embryos, however, do not develop directly into adults in this site. Gaining access to the circulation they are carried to the lungs and escape into the air spaces. Via the trachea they reach the pharynx and are swallowed. Partial development occurs during the above migration. It is completed in the small intestine, where egg-producing adults are found about 2½ months after infection.

Passage of the migrating larvae through the lung may produce severe bronchopneumonia. Larvae filtered out of the circulation in abnormal sites induce inflammatory lesions. Adult worms in the intestine often cause no symptoms. Particularly in heavy infections, however, intestinal and nervous disturbances may occur, and intestinal obstruction occasionally results. Massive infection of children in the tropics is common with numerous fatalities prior to the availability of piperazine. The drug acts as a myoneural blocking agent for Ascaris and the paralyzed worms are eliminated by normal peristalsis as they are no longer able to maintain their position in the intestine. Consequently, chemotherapeutic methods now are effective in infections which previously necessitated surgical intervention to eliminate intestinal obstruction by worms. Various other injuries result from migration of the adult worms out of the normal habitat into the appendix, the bile ducts, the upper alimentary or respiratory tracts, the genito-urinary system, or through the intestinal wall into the abdominal cavity. Hypersensitivity develops in infected persons and in laboratory workers who have handled the worms, and severe anaphylactic reactions may occur in such individuals.

Acquired immunity has not been observed in man, but experimental animals exhibit partial resistance to reinfection with related parasites. Antibodies are detectable by various tests after either infection or vaccination with worm extracts, and considerable investigation has been devoted to antigenic analysis. An allergic skin test has been used in diagnosis but is often positive in uninfected individuals who have acquired hypersensitivity from contact with the parasite. Laboratory diagnosis is preferably based on the finding of eggs in stools. However, a small proportion of cases harbor only male worms, and in such cases the immunological tests can be used.

Piperazine and hexylresorcinol are effective in removing the adult worms from the intestine. Some other drugs, particularly those used for treatment of hookworm infection, may cause migration of the adult Ascaris into abnormal sites.

Man is the only known host of *A. lumbricoides.* Infection predominates in children; that this results in part from age resistance is suggested by the marked age resistance of lower animals to related parasites. Infection is world-wide but most prevalent in the tropics and subtropics, where sanitary and climatic conditions favor its dissemination. Control depends on the reduction of soil

contamination by proper sewage disposal and treatment of infected individuals. Raw vegetables are an important source of infection and should be thoroughly washed before consumption.

Related species. Cats, dogs, cattle, horses, and other lower animals harbor a number of closely related species. Rare human infections have been reported with at least three of these. A distinct race of *A. lumbricoides* causes severe pneumonitis in young pigs, known as "thumps." In man the eggs hatch and the larvae reach the lung, but adult worms do not become established in the intestine. Similarly, the human *A. lumbricoides* develops only to the lung stage in pigs. The adults of the pig Ascaris are easily obtained from slaughterhouses for laboratory study.

Visceral larva migrans. Visceral larva migrans has been recognized only recently as a clinical entity and the nematodes responsible are close relatives of Ascaris.[10] The species most often involved are *Toxocara canis* and *T. cati*, the extremely common ascarids of the dog and cat.[97] The larvae do not mature in man but wander extensively through the tissues producing lesions and granulomata in the brain, eye, liver, lung, or other sites. The symptoms produced may be minor except for eosinophilia or may involve hepatomegaly, pulmonary infiltration, cough, hyperglobulinemia, and allergic manifestations. Diagnosis is difficult but may be made on recovery of larvae in sectioned biopsy material with recognition of the larvae as shown in the accompanying figure. Serologic reactions are of value in diagnosis but give cross-reactions with Ascaris.[72, 113] There is no specific therapy. Children with a history of dirt eating and an association with household pets run a risk of serious infection, emphasizing the need for anthelminthic treatment of dogs and cats.

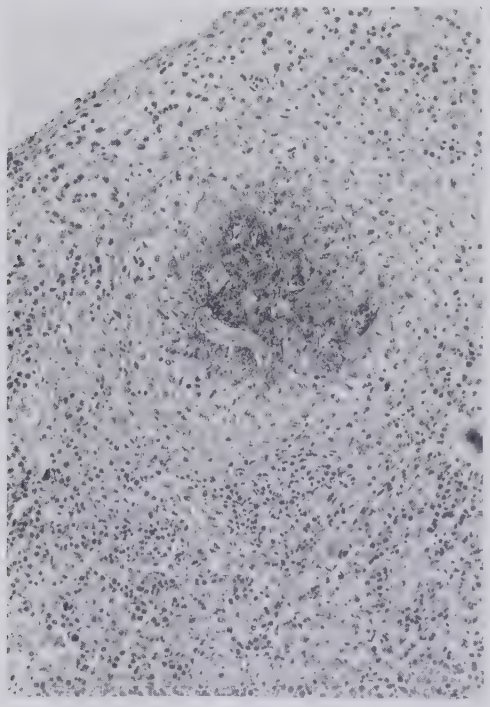


Figure 249. Human visceral larva migrans. Liver section showing granulomatous area containing *Toxocara canis* larva. × 105. (Beaver.)

Hookworms. The two important hookworms of man, *Ancylostoma duodenale* and *Necator americanus*, will be discussed together. Known since antiquity, human hookworms were first accurately described by Dubini in 1843. Their relationship to disease was definitely shown by Perroncito in 1880, and the life cycle was elaborated by Looss.

Characteristics and life cycle. The adults of *A. duodenale* from the human small intestine are shown in the accompanying figure. The female measures 1 to 1.5 cm. in length by about 0.5 mm. in diameter. The double reproductive system coils abundantly throughout the body cavity. Two pairs of prominent unicellular glands are seen in the anterior half of the body, one excretory in function, the other secreting histolytic enzymes. The dorsally directed mouth is characterized by two pairs of ventral teeth (replaced in *N. americanus* by curved cutting plates). The male measures a little less than 1 cm. in length by about 0.4 mm. in diameter. The reproductive system shows a long, muscular ejaculatory duct, a prominent seminal vesicle, and a coiled testis. At the posterior end of the male worm is a broad, flat clasping apparatus, the copulatory bursa, composed of cuticle supported by finger-like rays of tissue. *A. duodenale*

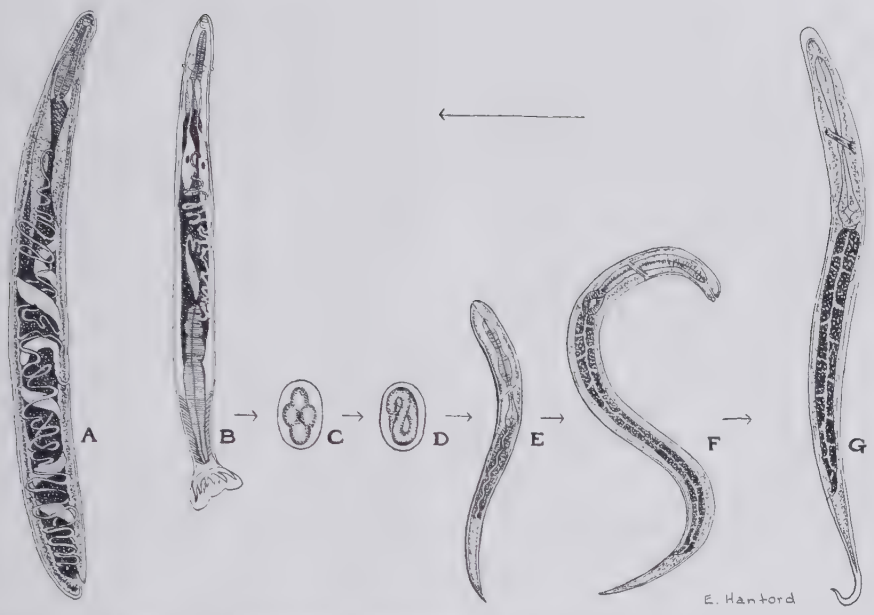


Figure 250. Life cycle of a hookworm, *Ancylostoma duodenale*. Note differences in magnification. *A*, female worm. *B*, male worm. *C* and *D*, eggs. *E*, first stage larva. *F*, second stage larva. *G*, infective larva. *A* and *B*; × 10; *C–G*; × 250. (Adapted from Looss.)

and *N. americanus* can be identified by details of structure of this bursa as well as by the differences in buccal teeth noted above.

The thin-walled eggs measure about 38 by 58 μ. Those of the two species of human hookworms cannot be distinguished microscopically from one another. They are usually in early stages of development upon escape from the body, maturing and hatching within 24 hours if conditions are favorable in the soil. The larva, about 0.25 mm. in length, feeds on fecal material and grows in 3 days or more to a length of about 0.4 mm. After molting it continues to grow until, by the fifth day or later, it measures about 0.6 mm. These two stages, characterized by a bulbous esophagus, are known as *rhabditoid larvae*. The third stage, resulting from a second molt, is a thinner form with a closed mouth and a less bulbous esophagus. It is usually still surrounded by a "sheath," the molted skin of the preceding stage. This is the *filariform* larva, the infective stage for man. It does not feed but may survive long periods in the soil under favorable conditions. If the infective larva comes in contact with human skin, it actively penetrates through the epidermis, reaches the circulation, and is carried to the lungs. Here, like the migrating larva of Ascaris, it breaks out into the air spaces and reaches the intestine via the trachea and esophagus. After two further molts, the worms are mature. Egg production begins about 1 month after infection.

Hookworm disease. Penetration of the skin by hookworm larvae produces a dermatitis known as ground itch or miner's itch. This is severe enough in some previously exposed individuals to be incapacitating. The adult hookworms live attached to the mucosa of the small intestine, feeding on blood and bits of tissue. They move about frequently, leaving bleeding wounds in the intestinal mucosa. The chronic loss of blood causes anemia and edema, resulting, in severe cases, in retardation of growth and mental development and in weakness and general debilitation.[44] Intestinal disturbances are seen in heavy infections. Death, except in infants and young children, is rarely a direct result of hookworm disease, but the condition often contributes to death from other causes. In white persons the symp-

toms are directly related to the intensity of infection, which, because of the necessary development outside the body, is stable unless re-exposure occurs. Individuals with fewer than twenty-five *N. americanus* virtually never show symptoms. Those with between twenty-five and 100 worms show borderline effects. With more than 100 worms some clinical injury is almost always detectable. *A. duodenale* is more harmful, about half as many worms being required to produce a given effect. Normally, infection is maintained by constant re-exposure. In previously infected persons living under conditions which prevent additional infection, however, it has been shown that about one-half of the worms are lost in 6 months and about three-quarters in 2 years. A smaller number persist for as much as 12 years.

Immunity. Puppies can be largely protected against the dog hookworm by repeated small infections. Similar partial protection has been reported in a human volunteer. That antibodies are involved is shown by the fact that some protection is conferred on puppies by immune serum.

Diagnosis. Diagnosis of hookworm infection is based on recovery of characteristic eggs from the feces. Direct fecal smears were formerly used, but many infections with fewer than twenty-five worms are not detected with this method. Because the number of hookworms present in an infected individual is so important, several quantitative diagnostic methods have been devised. In the Stoll method a measured quantity (4 ml.) of feces is mixed with dilute NaOH solution (56 ml.), and all the eggs in a known sample volume (0.075 ml.) of this suspension are counted. The Lane method, or D.C.F. (direct centrifugal flotation), can be applied to lighter infections. One milliliter of feces washed by centrifugation is mixed with brine and recentrifuged with a coverslip on top of the tube. The eggs rise and can be counted on the coverslip. A relatively simple and accurate densitometric technique has also been devised.[8] Figures obtained by either of these methods are expressed as the number of eggs per milliliter of feces. A single female *N. americanus* produces about forty-five eggs per ml. of feces (about 6000 eggs per day) so that twenty-five worms (male and female) produce about 600 eggs per ml. and 100 worms produce about 2500 eggs per ml. *A. duodenale* produces about twice as many eggs per worm, but because of the greater pathogenicity of this species the egg output gives a roughly equivalent index of the severity of infection in both species.

A number of drugs are effective in eliminating hookworms from the intestine. The best at present are tetrachlorethylene and hexylresorcinol.

Epidemiology and control. Worms morphologically similar to *N. americanus* occur in pigs, but infection experiments indicate that they are distinct from the human hookworms. Man is the reservoir of infection with both *A. duodenale* and *N. americanus.*

There has been much study of the habits and requirements of hookworm larvae in the soil. The optimal temperatures are about 75° F. for *A. duodenale* and about 80° F. for *N. americanus.* Consistent high temperatures, about 100° F., are unfavorable. At 60° F. development is prolonged to 2 weeks or more, and below 50° F. there is little or no development. Continued temperatures below 40° F. kill the larvae. Moisture is essential to survival of the soil stages, but they die under water and are scattered by heavy rain. The type of soil is significant, probably largely in relation to its water-holding properties. Coarse sand and heavy clay are unfavorable, optimal development occurring in light sand or sandy loam. A large proportion of the infective larvae die within the first 2 weeks, but a few survive for several months under favorable conditions. Although lateral migration from the site of development is insignificant, the filariform larvae can move considerable distances vertically in the soil. Unless excessive drying occurs, they remain at the surface, where the chance of contact with human skin is greatest.

In addition to the environmental re-

quirements of the larvae, various human factors affect the distribution of hookworm infection. Fecal disposal practices and the use of shoes are among the most important. All these factors combine to make hookworm infection a problem of the rural parts of warm countries. Exceptions occur in mines and tunnel-building operations, where local conditions may be favorable despite a generally unsuitable environment. *N. americanus* is the hookworm of central and southern Africa, the Western Hemisphere and southern India. Throughout the rest of southern Asia it is mixed with *A. duodenale*, and a few foci of *A. duodenale* occur in South America. In most of Asia, southern Europe, European mines, and North Africa only *A. duodenale* is found.

In the southern United States hookworm infection is most common in children, largely because adults are protected from infection by wearing shoes. That age resistance, if it exists at all, is a minor factor is shown by the fact that in countries where the whole population goes without shoes infection may predominate in adults. Racial immunity is marked, and Negroes under comparable conditions have fewer and less intense infections than do whites. Furthermore, Negroes seldom show clinical symptoms except in the presence of heavy infections.

Control depends on the reduction of soil contamination by treatment of cases and proper fecal disposal and on the prevention of contact between human skin and the soil. Chemical destruction of larvae in the soil has been of use only in particular cases, such as mines. Education is the major weapon in hookworm control, for the chief problems are sociological, and biological knowledge of the parasite is adequate to insure successful hookworm eradication if the habits of infected populations can be sufficiently influenced. Treatment of cases has an important place in hookworm campaigns. Formerly it was customary to treat all infected members of a community, or even the whole community (mass treatment), if sample diagnoses showed abundant and heavy infections. Current opinion favors treatment of only the clinically significant cases. In most regions the treatment of a small proportion of the population can eliminate most of the egg production.

Related nematodes of lower animals. Various animals harbor hookworms. Two species, *Ancylostoma braziliense* and *A. caninum* of dogs and cats, occasionally invade the skin of man. They are unable to develop further, and their wandering in the subcutaneous tissues produces a serpiginous dermatitis known as creeping eruption or cutaneous larva migrans. The adults of *A. braziliense* have been reported from man in the Far East. It has been claimed that the human cases harbor a distinct species, *A. ceylonicum*. Other, more distantly related parasites such as the numerous species of Trichostrongylus are disease agents of ruminants and are incidental parasites of man. One species, *T. orientalis*, is more common in man than in other animals. Heavy infections in man may produce diarrhea and high eosinophilia. The eggs resemble those of the hookworms but may be distinguished from them by their larger size and slightly different shape. The rat lungworm, *Angiostrongylus cantonensis*, has been found in human brain where it causes a parasitic eosinophilic meningo-encephalitis.[3] In Asia and the Pacific islands this parasite is transmitted by the ingestion of raw land snails and slugs which are the intermediate hosts. It has been suggested that this organism is the cause of the "epidemic" eosinophilic meningitis of Tahiti.[109]

Nippostrongylus brasiliensis (*muris*) of rats and mice is of interest because of extensive studies in the laboratory. Serum antibodies have been shown to exert a direct action on the parasites *in vitro*, and these antibodies together with tissue cells immobilize and destroy the larvae in the skin and lungs and destroy adults in the intestine.

The first true cultivation of a parasitic worm has recently been accomplished with *N. brasiliensis* and *Nematospiroides dubius*.[139] Partial development

of *Necator americanus* has been obtained. In a complex mixture of chick embryo extract, vitamins, amino acids, minerals, etc., the whole development of *N. brasiliensis* from the egg to the adult stage has been observed. While normal male and female worms were produced, the eggs produced were infertile. It is to be hoped that exploitation of this line of research will provide much needed information about parasitic worms.

Studies have been made of the mechanisms involved in the exsheathment or cuticle shedding necessary between developmental stages of nematodes. The fluids responsible for this are believed important in stimulating the antibodies producing functional immunity to several of these infections. Extensive studies on the development of living nematode vaccines for use in veterinary medication are in progress, as in some forms it is believed that only antigenic stimulation by such metabolic products can produce significant immunity.[39, 122]

Strongyloides Stercoralis. *Strongyloides stercoralis* is a common intestinal parasite of man in warm countries. It exhibits a life cycle of extreme complexity, involving reproduction by adults in the soil as well as in the human intestine. Investigators working with different species of Strongyloides disagree on fundamental points, and it is not clear whether or not these disagreements result from specific differences in the worms studied.

Only female worms are known in the intestine of man. They are thin, transparent worms 2 to 2.5 mm. long by about 0.05 mm. in diameter. They lie buried in the mucosa of the small intestine, and the thin-shelled eggs are deposited in the tissues, where they hatch to release rhabditiform larvae very similar to those of hookworms. Escaping in the feces the larvae undergo one of two types of development. They may molt to filariform larvae which penetrate the skin to establish infection. Alternatively, they may develop into the free-living males and females. The free-living female is stouter than the parasitic female and about 1 mm. long. The male is somewhat smaller, about 0.7 mm. in length. Eggs produced by the free-living female hatch to release rhabditiform larvae which may continue the free-living cycle or may develop into infective larvae. The infective larvae, whether produced

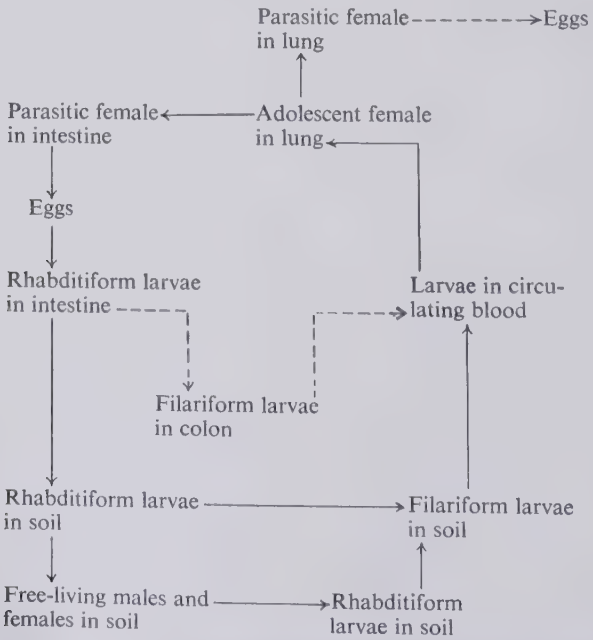


Figure 251. Schematic life cycle of *Strongyloides stercoralis*. Broken arrows indicate rare or incompletely understood possibilities of development.

by free-living adults or developed directly from the rhabditiform larvae in the feces, penetrate the skin of man and reach the intestine via the circulation and lungs as in the hookworms. Male worms have not been found in the intestine, but "parasitic" males like those in the soil have been reported in the lung in abnormal hosts. Since investigation of a related species in rats has shown that a single infective larva can give rise to a female in the intestine producing fertile eggs, it is clear that the parasitic males are not essential in all species of Strongyloides. Oviposition in the lung has also been reported. A final complication is introduced by the reports that rhabditiform larvae might transform into infective larvae in the colon and penetrate its wall or the perianal skin to produce hyperinfections. The various paths of development indicated above are shown in the accompanying diagram, the dotted lines indicating phenomena which are incompletely known.

Penetration of the skin by the larvae causes dermatitis, and passage through the lung may give rise to bronchopneumonia. The parasitic females cause intestinal disturbances characterized chiefly by diarrhea. Laboratory diagnosis is based on identification of the rhabditiform larvae in the feces. These can be distinguished by the shorter buccal cavity from the rhabditoid larvae of the hookworms, which are occasionally encountered in fecal specimens. Dithiazanine is the most effective drug for therapy of *S. stercoralis* although not completely efficient in eliminating infection.

The epidemiology of *S. stercoralis* infection is similar to that of hookworm, and the same sanitary control measures apply. Dogs are infected with a strain indistinguishable from the form found in man and may be of some consequence in its maintenance. *S. ratti* of rats is a similar form which can produce local allergic manifestations in man and cutaneous eruptions similar to those of the hookworms.

Trichinella Spiralis. The "trichina" worm, *Trichinella spiralis,* was first seen in human muscles at necropsy by Tiedemann in 1821. Leuckart and others worked out the life cycle.[55] The adult forms are intestinal parasites of man and many other mammals, but their sojourn in the intestine is so brief that the more persistent larval infection in the muscles receives the main emphasis. The infective stage for man is a larva in the muscles of the hog. This stage is a minute coiled worm, about 1 mm. in length, enclosed in a lemon-shaped fibrous cyst, 0.25 by 0.5 mm. in dimensions. Ingested by a susceptible host the cysts reach the stomach, where the muscle and cyst wall are digested away. In about 2 days the larvae mature in the small intestine to adult males and females. The male is a thin, transparent worm about 1.5 by 0.04 mm. (Fig. 252). The anterior half of the body is occupied by a nonmuscular esophagus like that of the closely related *Trichuris trichiura.* At the posterior end are two pear-shaped clasping lobes. The female is about 4 by 0.06 mm. in size. After fertilization the females burrow into the mucosa where, after about 1 week, they begin to deposit minute larvae, about 0.1 mm. in length, in the tissues. These larvae reach the general circulation and are filtered out in the skeletal muscles, cardiac muscle, the central nervous system, and other sites. In most tissues they die, but in skeletal muscle they grow in about 2 weeks to the infective stage. One month after infection the larvae are surrounded by a fully developed cyst wall, produced by the host tissues. Much later, probably only after death of the larvae, these cysts become calcified, but they are known to remain infective at least for several months. Any skeletal muscle may be infected, but the larvae are most abundant in the diaphragm, intercostals, tongue, larynx, and eye muscles.

Meanwhile the adults have disappeared from the intestine. Most of the males die and are passed out within a week after infection. The females have largely disappeared within a month. It will be seen that the complete life cycle occurs in a single individual, for infec-

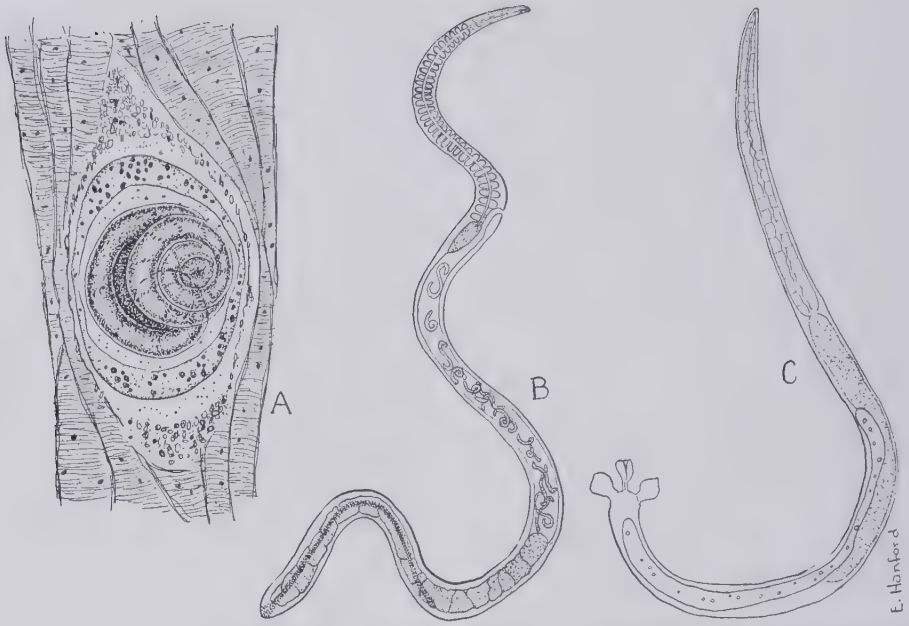


Figure 252. *Trichinella spiralis*. *A*, larva in muscle; × 100. *B*, adult female; × 50. *C*, adult male; × 50.

tive larvae are produced in the tissues of the same host which harbors the adults in its intestine.

Trichinosis. Although the incidence of infection in a population may be high, the usual infection with Trichinella is without detectable symptoms and the damage to the host is probably insignificant. However, severe cases resulting from relatively massive infection occur and are characterized by two phases. Intense infection with the adult worms produces gastrointestinal disturbances, usually with diarrhea. Occasionally the injury to the intestine is so severe as to result in death within a few days after infection. Muscle invasion by the larvae leads to varied signs of toxemia, hypersensitivity, and muscle injury. Heavy infections cause death or chronic illness. The severity of the disease is proportional to the number of larvae ingested, and many mild cases undoubtedly escape detection.

Immunity to superinfection is demonstrable in experimental animals and can be transferred with immune serum. Some protection may be produced in the laboratory by vaccination with killed larvae, but this is insufficient to be of practical value. Direct action of immune serum on the worms has been described. Precipitin and skin tests are available using extracts of larvae digested out of infected muscle. These reactions are detectable 2 to 3 weeks after infection and remain positive for several years. Slide flocculation tests with antigen coated on bentonite particles or on cholesterol are the preferred immunological reactions for diagnosis. Like the complement-fixation test they become negative a few months after infection and are thus indicative of current or recent trichinosis. The immunological and serological aspects of trichinosis have been the subject of a recent review which summarizes the numerous techniques which have been employed in diagnosis.[73] Eosinophilia, often over 20 per cent, is characteristic and offers presumptive indication of trichinosis. Other diagnostic methods involve search for the larvae in body fluids or tissues. During migration they may be found occasionally in blood or spinal fluid, but it is often impossible to detect them. Muscle biopsies show the larvae in severe cases. There is no specific treatment for trichinosis.

T. spiralis occurs principally in man, hogs, rats, and bears, although experimental infection has been produced in

many species of mammals and birds. Human infection follows the consumption of undercooked pork (more rarely the meat of other animals, such as bears), while pigs acquire the parasite from garbage containing pork scraps or, less commonly, by eating carcasses of infected rats. Infection through the ingestion of larvae which pass out in feces has also been demonstrated and may be of some importance in the infection of coprophagous swine. In the United States as well as in the Arctic regions a significant sylvatic infection in carnivores exists. *T. spiralis* is world-wide in distribution, though infection in the tropics is rare. In the United States routine necropsy examination of human diaphragms has revealed a general infection rate of between 14 and 28 per cent despite the fact that very few clinical cases are diagnosed.

Personal protection can be assured by thorough cooking of pork products, including hamburger of unknown composition. Government meat inspection does not attempt to detect Trichinella, for examination guaranteeing safety is considered impractical, but refrigeration for 24 hours at —18° C. destroys most of the larvae. Laws which forbid the feeding of raw garbage to hogs have been successful in reducing the incidence of infection. New methods of food preservation with gamma radiation show promise for control of trichinosis. Treatment of meat with radioactive cobalt renders the worms infertile, so that they cannot produce larvae. This prevents not only the systemic effects but also the intestinal pathology of early trichinosis.

The Filaria

Seven species of nematodes belonging to this group are important parasites of man. All produce motile embryos termed microfilariae which are produced in large numbers by the female in the host tissues and are released within a sheath (believed to be an elongate eggshell) or unsheathed. Microfilariae migrate or are carried by the circulation to the skin or to peripheral vascular beds where they may be ingested by blood-sucking arthropods. Within this insect intermediate host the microfilariae transform without multiplication into infective larvae, become associated with the insect mouthparts, and may then be transmitted by the insect to the definitive host. After an incubation period and growth, which is little understood and may take as much as a year, the filaria may be found in their final sites of parasitism and are producing larvae. *Wuchereria bancrofti* and *Brugia malayi* adults parasitize lymphatic vessels; *Onchocerca volvulus* is usually in subcutaneous nodules or in deeper connective tissue as is *Acanthocheilonema streptocerca. Mansonella ozzardi* and *Acanthocheilonema perstans* are found in pleural or peritoneal cavities, while *Loa loa* as an adult migrates extensively through the connective tissues.

Wuchereria Bancrofti. Of the several species of filaria mentioned above, *Wuchereria bancrofti* is by far the most important. The disease, elephantiasis, was known in antiquity, but the larvae were first seen in 1863 by Demarquay and the adults by Bancroft in 1876. Manson, in 1878, showed that development of the larvae took place in mosquitoes. This discovery is noteworthy because it represents the first incrimination of an insect in the transmission of disease.

Characteristics and life cycle. The adults live coiled in the lymph nodes of man. They are thread-like translucent worms, the female 7 to 10 cm. long by 0.25 mm. in diameter, the male 4 cm. by 0.1 mm. The egg, covered by a thin membrane, hatches in the uterus or in the host tissues to release an active embryo about 0.2 mm. long. This embryo, known as a *microfilaria,* escapes into the lymph and enters the circulating blood by way of the thoracic duct. In most regions the embryos of *W. bancrofti* show marked nocturnal periodicity, occurring in the peripheral blood almost exclusively at night. In many of the South Pacific islands, however, they appear in the blood at all hours. Extensive

Figure 253. Microfilaria of *Wuchereria bancrofti* in thick blood film. × 300.

study has failed to provide a satisfactory explanation of this periodicity, although there is suggestive evidence that daytime activity induces accumulation of the microfilariae in capillaries of the lung.[58]

The microfilaria develops no further in the circulating blood. If ingested by a susceptible mosquito, however, it undergoes a series of transformations in the tissues of the insect. Within a day the larva leaves the stomach of the mosquito and invades the thoracic muscles. Here it develops into a stout "sausage larva," which, after a second molt, elongates to form the infective stage, a thin worm 1.5 to 2 mm. in length. This infective larva leaves the muscles and migrates to the proboscis sheath of the mosquito, arriving 6 days or more after the infective blood meal. When the mosquito takes a new blood meal, the infective larva, apparently stimulated by the warmth of the skin, breaks out of the proboscis sheath and penetrates the skin, probably through the wound caused by the biting mosquito, to establish infection in man. The early development of the worms in man is not known, but microfilariae appear in the circulating blood several months after infection. In the related species, *Brugia malayi* (see below), development to the adult stage requires about 80 days in experimental animals. Although the longevity of the adult worms in the lymphatics has not been determined, they probably live for several years.

Filariasis. The adult worms are most prevalent in the lymph nodes of the inguinal region, but they are also found in lymphatics elsewhere in the body. They induce inflammation and fibrosis in the infected nodes. The consequent restriction of lymph flow causes edema, lymphangitis, lymphadenitis, and elephantiasis. Secondary infection of the lymphatics with streptococci and staphylococci is often significant. Long-standing cases sometimes show enormous enlargement of the scrotum, vulva, legs, or breasts.

Evidence of acquired immunity is lacking, but complement-fixing antibodies are detectable in the serum, and skin tests are positive in infected individuals. The reactions are group-specific, occurring with extracts of filarial worms from lower animals and giving false positives in other nematode infections. Laboratory diagnosis is preferably based on the identification of microfilariae in fresh blood preparations or stained thick blood films. They are often not detectable in early infections or in long-standing cases with much tissue damage. Other filarial worms infecting man (see below) also produce microfilariae in the circulating blood, but these can be differentiated microscopically from the larvae of *W. bancrofti*.

Certain antimony compounds, notably Neostibosan and Anthiomaline, eliminate microfilariae from the blood of cases for long periods. Hetrazan, an oral drug, is more effective and less toxic and has been widely used, principally because of its ease of administration and its ability to minimize mosquito infec-

tions by eliminating microfilariae in the human reservoir. It is not known whether these drugs kill the adult worms in man, but they destroy the adults of other filarial worms in experimental animals.

Epidemiology and control. Man is the only known host of the adult worm. A large number of mosquitoes of the genera Culex, Aedes, Mansonia, and Anopheles support development of the larvae, but the most important are *Aedes polynesiensis* in the South Pacific islands, *Anopheles gambiae* in West Africa, and *Culex quinquefasciatus* (*C. fatigans*) elsewhere. There is an interesting correlation between the habits of the mosquito hosts and the periodicity of the microfilariae discussed above. *Aedes polynesiensis* bites by day, and it is in the regions where this mosquito transmits *W. bancrofti* that the microfilariae are found in the peripheral blood at all hours. In other regions, where the principal vectors attack man in the evening or at night, the microfilariae show a marked nocturnal periodicity.

W. bancrofti is a parasite of warm countries, occurring widely throughout the tropics and subtropics. Climate favors the production of mosquitoes and the development of larvae of *W. bancrofti* in the insect, and the poorer housing of the tropics permits greater contact between mosquitoes and man. The slow development of human infections, the lack of multiplication of *W. bancrofti* in the mosquito hosts, and other hazards combine to limit dissemination of the parasite. As a consequence the infection seems to die out in an area unless there are many human cases and abundant mosquitoes. The former endemic center in the United States, around Charleston, South Carolina, where the incidence was 20 to 30 per cent before 1920, now shows no cases. Control is a matter of mosquito reduction, protection from mosquito bites, attention being directed to the habits of the particular vectors in any area, and chemotherapy of cases.

Other Species of Filarial Worms. *Brugia malayi* is very similar to *W. bancrofti* and is widely distributed in southeast Asia, the China Sea area, and Eastern India, often occurring with *W. bancrofti.* In some areas it is transmitted by mosquitoes of the genus Mansonia whose larvae obtain oxygen from subaquatic vegetation, posing additional problems of control. A similar parasite, *B. pahangi,* of Malayi is common in wild and domestic mammals and, in man, may produce the symptoms of tropical eosinophilia.

Onchocerca volvulus is widely distributed through central Africa, and in the Western Hemisphere has been introduced into Guatemala, Mexico, Venezuela, and Surinam.[107] The adults are found in tightly coiled masses in subcutaneous nodules of connective tissue where commonly they may be extirpated by minor surgery. Others may be deep-seated and not palpable. The microfilariae are not found in the circulating blood but concentrate in the skin where they may readily be demonstrated by superficial biopsy. In the Western Hemisphere nodules containing the adults are found on the head, and the microfilaria cause ocular lesions commonly leading to blindness. Sensitization reactions are usual in infection with Onchocerca and may be pronounced following chemotherapy with Hetrazan.

Blackflies of the genus Simulium, whose larvae must develop in fast-flowing mountain streams, serve as intermediate hosts. Since man is the only known definitive host, attempts have been made to provide control by surgical removal of the onchocercal nodule plus chemotherapy. Intravenous Suramin (Naphuride sodium) is effective.

Loa loa is found in the subcutaneous tissues of a variety of primates including man and is limited to West and Central Africa where it is transmitted by the biting deerfly Chrysops. It has been called the "eye worm" because of the frequency with which it may be found passing under the conjunctiva of the eye surface, and it is also associated with a fugitive "calabar" swelling presumed to be due to reaction of the host to the metabolic products of the migrating

adult worm. The microfilariae are diurnal, in the peripheral circulation, and unsheathed.

Acanthocheilonema streptocerca is found in the dermal and subcutaneous connective tissue and its unsheathed microfilariae are in the superficial layers of the dermis. It is limited in distribution to parts of Central Africa. *A. perstans* has a wide distribution in tropical Africa and is also present in coastal areas of South America and the West Indies. Its microfilariae are nonperiodic, unsheathed, and are found in the peripheral blood. The adults are found associated with the peritoneal cavity, in mesentery, in retroperitoneal tissues, in the pericardium, or occasionally in subcutaneous cysts. Infections are often without symptoms but may be associated with sensitization phenomena and rarely with more serious or fatal complications. *Mansonella ozzardi* is also found in the tropical areas of the Western Hemisphere, occupies body cavities, and has circulating, unsheathed, nonperiodic, microfilariae. No symptoms are associated with infection. Both species of Acanthocheilonema and Mansonella are transmitted by gnats of the genus Culicoides.

There are numerous filarial worms of mammals and birds widely distributed over the earth. One of these *Dirofilaria immitis,* is an important pathogen of dogs, the adult worms living in and occluding the vessels of the heart. Antigen prepared from Dirofilaria has been used in intradermal and serological tests for the diagnosis of filariasis in man. The specificity of the reactions is low.[1] *Litomosoides carinii,* a parasite of the body cavity of the cotton rat, is a form commonly used in laboratory investigations.

Dracunculus medinensis, the Guinea worm, is a common parasite of man in the Middle East, Africa, India and Indonesia. It was also endemic in the tropics of the Western Hemisphere but has not been recently reported. Although a tissue parasite resembling the filaria, it does not have a true microfilarial stage. The small male worms are rarely seen. The females, after several months' development in internal connective tissues, appear in the subcutaneous tissues, usually of the leg. They are large worms, averaging about 1 meter in length, often visible through the skin as they lie in the tissues. The skin of the host ulcerates at the anterior end of the worm, and larvae escape, usually when the leg is submerged in water. These larvae develop in copepods, *e.g.,* cyclops, and human infection follows ingestion of the parasitized copepods in drinking water. The human infection, possibly the "fiery serpent" of the Bible, is associated with severe sensitivity reactions immediately preceding initial blister formation and larval release by the female. In some regions the female worm is gradually removed from the tissues by rolling it up on a stick. Local injection of phenothiazine is said to be effective in therapy and antihistamines may be used to relieve the sensitivity phenomena.

REFERENCES

1. Adolph, P. E., Kagan, I. G., and McQuay, R. M.: Diagnosis and treatment of *Acanthocheilonema perstans* filariasis. Amer. J. Trop. Med. Hyg., 1962, **11:**76–88.
2. Africa, C. M., et al.: Visceral complications in intestinal heterophyidiasis of man. Acta Med. Philippina Monograph Ser. No. 1, 1940.
3. Alicata, J. E.: *Angiostrongylus cantonensis* (Nematoda: Metastrongylidae) as a causative agent of eosinophilic meningoencephalitis of man in Hawaii and Tahiti. Can. J. Zool., 1962, **40:**5–8.
4. Alving, A. S., et al.: Observations on Primaquin in the prophylaxis and cure of vivax malaria. Proc. Sixth Int. Cong. Trop. Med. Malaria, Lisbon. 1959.
5. Arean, V. M., and Koppisch, E.: Balantidiasis: a review and report of cases. Amer. J. Path., 1956, **32:**1089–1115.
6. Aufses, A. H., et al.: Portal venous pressure in "pipestem" fibrosis of the liver due to schistosomiasis. Amer. J. Med., 1959, **27:**807–810.
7. Baer, J. G.: Ecology of Animal Parasites. University of Illinois Press. 1951.
8. Beaver, P. C.: The standardization of fecal smears for estimating egg production and worm burden. J. Parasit., 1950, **36:**451–456.
9. Beaver, P. C., et al.: Experimental *Entamoeba histolytica* infection in man. Amer. J. Trop. Med. Hyg., 1956, **5:**1000–1009.
10. Beaver, P. C.: Visceral and cutaneous larval migrans. Pub. Health Repts., 1959, **74:**328–332.
11. Beye, H. K., et al.: Simian malaria in man. Amer. J. Trop. Med. Hyg., 1961, **10:**311–316.

12. Bishop, A.: Drug resistance in protozoa. Biol. Rev., 1959, **34**:445–500.
13. Black, R. H.: Blood donors in accidental transfusion malaria. Med. J. Australia, 1960, **2**: 446–449.
14. Boyd, M. F. (ed.): Malariology. W. B. Saunders Co., Philadelphia. 1949.
15. Bray, R. S.: Studies on the exoerythrocytic cycle in the genus Plasmodium. London School of Hygiene and Tropical Medicine, Memoir 12. Lewis, London. 1957.
16. Brooke, M. M.: Amebiasis, Methods in Laboratory Diagnosis. Public Health Service, U. S. Department of Health, Education & Welfare. 1958.
17. Brown, H. H.: The actions and uses of anthelmintics. Clin. Pharm. Therapeutics, 1960, **1**: 87–103.
18. Bueding, E., and Swartzwelder, C.: Anthelmintics. Pharm. Reviews, 1957, **9**:329–365.
19. Bueding, E.: Mechanisms of action of schistosomicidal agents. J. Pharmacy Pharmacol., 1959, **11**:385–392.
20. Burch, T. A., Rees, C. W., and Reardon, L. V.: Epidemiological studies on human trichomoniasis. Amer. J. Trop. Med. Hyg., 1959, **8**:312–318.
21. Burrows, R. B., and Swerdlow, M. A.: *Enterobius vermicularis* as a probable vector of *Dientamoeba fragilis*. Amer. J. Trop. Med. Hyg., 1956, **5**:258–265.
22. Burrows, R. B.: Morphological differentiation of *Entamoeba hartmanni* and *E. polecki* from *E. histolytica*. Amer. J. Hyg., 1959, **8**:583–589.
23. Campbell, D. H., and Melcher, L. R.: Relationships of sex factors to resistance against *Cysticercus crassicollis* in rats. J. Infect. Dis., 1940, **66**:184–188.
24. Chandler, A. C., and Read, C. P.: Introduction to Parasitology. John Wiley and Sons, New York. 1961.
25. Chitwood, B. G., and Chitwood, M. B.: An introduction to Nematology. Monumental Printing Co., Baltimore. 1950.
26. Chu, G. W. T. C.: Pacific area distribution of fresh-water and marine cercarial dermatitis. Pacific Science, 1958, **12**:299–312.
27. Coatney, G. R., Alving, A. S., et al.: Korean vivax malaria. Amer. J. Trop. Med. Hyg., 1953, **2**:958–988.
28. Coatney, G. R., et al.: Chloroquin or pyrimethamine in salt as a supressive against sporozoite-induced vivax malaria (Chesson strain). Bull. W. H. O., 1958, **19**:53–67.
29. Contacos, P. G., et al.: Man to man transfer of two strains of *Plasmodium cynomolgi* by mosquito bite. Amer. J. Trop. Med. Hyg., 1962, **11**:186–193.
30. Cort, W. W.: Studies on schistosome dermatitis. Amer. J. Hyg., 1950, **52**:251–307.
31. Cortner, J. A.: Giardiasis, a cause of celiac syndrome. Amer. J. Dis. Children, 1959, **98**: 311–316.
32. Davey, D. G.: Human and animal trypanosomiasis in Africa. Amer. J. Trop. Med. Hyg., 1958, **7**:547–553.
33. Deschiens, R.: Distomatose cérébrale à *Heterophyes heterophyes*. Abstr. 6th Int. Cong. Trop. Med. Malaria, 1958, p. 265.
34. Diamond, L. S.: Axenic cultivation of *Entamoeba histolytica*. Science, 1961, **134**:336–337.
35. Dunning, W. F., and Curtis, M. R.: Attempts to isolate the active agent in *Cysticercus fasciolaris*. Cancer Res., 1953, **13**:838–842.
36. Elsdon-Dew, R.: Further aspects of amebiasis in Africans. African J. Med., 1956, **2**:291–294.
37. Eyles, D. E.: The exoerythrocytic cycle of *Plasmodium cynomolgi* and *P. cynomolgi bastianelli* in the rhesus monkey. Amer. J. Trop. Med. Hyg., 1960, **9**:543–555.
38. Eyles, D. E., and Gibson, C. L.: Toxoplasma infections in animals associated with a case of human congenital toxoplasmosis. Amer. J. Trop. Med. Hyg., 1957, **6**:990–1000.
39. Fairbairn, D.: Physiologic aspects of egg hatching and larval exsheathment in nematodes. In Stauber, L. E. (ed.): Host Influence on Parasite Physiology, pp. 50–64. Rutgers University Press, 1960.
40. Faust, E. C., Beaver, P. C., and Jung, R. C.: Animal Agents and Vectors of Human Disease. Lea & Febiger, Philadelphia. 1962.
41. Faust, E. C., and Russell, P. F.: Clinical Parasitology. Lea and Febiger, Philadelphia. 1957.
42. Faust, E. C. et al.: Human isoporiasis in the Western Hemisphere. Amer. J. Trop. Med. Hyg., 1961, **10**:343–349.
43. Field, J. W., and Le Fleming, H.: The morphology of malarial parasites in thick blood films. Trans. Roy. Soc. Trop. Med. Hyg., 1938, **32**: 467–480; 1939, **33**:507–520; 1940, **34**:297–304.
44. Foster, A. C., and Landsberg, J. W.: The nature and cause of hookworm anemia. Amer. J. Hyg., 1934, **20**:259–290.
45. Freedman, L.: The epidemiology of amebiasis in Durban. S. African Med. J., 1958, **32**:797–798.
46. Frenkel, J. K.: The pathogenesis and management of toxoplasmosis. World Neurol., 1961, **2**:1046–1068.
47. Fulton, J. D.: Some aspects of research on trypanosomes. In Stauber, L. E. (ed.): Host Influence on Parasite Physiology, pp. 11–23. Rutgers University Press, 1960.
48. García-Palmieri, M. R., and Marcial-Rojas, R. A.: Portal hypertension due to schistosomiasis mansoni. Amer. J. Med., 1959, **27**:811–816.
49. Garnham, P. P. C., and Lewis, D. J.: Parasites of British Honduras with special reference to leishmaniasis. Trans. Roy. Soc. Trop. Med. Hyg., 1959, **53**:12–40.
50. Geiman, Q. M., et al.: Studies on malarial parasites. VII. Methods and techniques for cultivation. J. Exptl. Med., 1946, **84**:583–606.
51. Gelfand, M.: Schistosomiasis in South Central Africa. Juta, Capetown. 1950.
52. Goble, F. C.: A comparison of strains of *Trypanosoma cruzi* indigenous to the United States with certain strains from South America. Proc. 6th Int. Cong. Trop. Med. Malaria, 1958, **3**:158–166.
53. Goldman, M.: Use of fluorescein-tagged antibody to identify cultures of *Endamoeba histolytica* and *Endamoeba coli*. Amer. J. Hyg., 1954, **59**:318–325.
54. Goldman, M., et al.: Antigenic analysis of *Entamoeba histolytica* by means of fluorescent antibody. II. *E. histolytica* and *E. hartmanni*. Exptl. Parasit., 1960, **10**:366–388.
55. Gould, S. E.: Trichinosis. Thomas, Springfield, Illinois. 1945.
56. Greenberg, J.: Mixed lethal strains of *Plasmo-*

dium gallinaceum: drug-sensitive, transferable (SP) × drug-resistant, non-transferable (BI). Exptl. Parasit., 1956, **5:**359–370.
57. Hackett, L. W.: Malaria in Europe. Oxford University Press, 1937.
58. Hawking, F., and Thurston, J. P.: The periodicity of microfilariae. I. The distribution of microfilariae in the body. II. The explanation of its production. Trans. Roy. Soc. Trop. Med. Hyg., 1951, **45:**307–340.
59. Herms, W. B.: Medical Entomology. The Macmillan Co., New York. 1961.
60. Hoare, C. A.: The enigma of host-parasite relations in amebiasis. In Symposium: Resistance and immunity in parasitic infections. The Rice Institute Pamphlet, 1958, **55:**23–35.
61. Hoare, C. A.: Amoebic infections in animals. Vet. Rev. Annot., 1959, **5:**91–102.
62. Hoeppli, R.: Parasites and Parasitic Infections in Early Medicine and Science. University of Malaya Press, 1959.
63. Honey, R. M., and Gelfand, M.: The Urological Aspects of Bilharziasis in Rhodesia. Livingston, Edinburgh. 1960.
64. Honigberg, B. M., and Read, C. P.: Virulence transformation of a trichomonad protozoan. Science, 1960, **131:**352–353.
65. Horsfall, W. R.: Medical Entomology: Arthropods and Human Disease. The Ronald Press, New York. 1962.
66. Hsu, H. F., and Hsu, S. Y. Li: New approach to immunization against *Schistosoma japonicum.* Science, 1960, **133:**766.
67. Huff, C. G., and Bloom, W.: A malarial parasite infecting all blood and blood-forming cells of birds. J. Infect. Dis., 1935, **57:**315–336.
68. Huff, C. G., et al.: The morphology and behavior of living exoerythrocytic stages of *Plasmodium gallinaceum* and *P. fallax* and their host cells. J. Biophys. Biochem. Cytol., 1960, **7:**93–102.
69. Hull, T. G.: Diseases Transmitted from Animals to Man. Thomas, Springfield, Illinois. 1962.
70. Jaramalinta, R., and Maegraith, B. G.: Hyaluronidase activity in stock cultures of *Entamoeba histolytica.* Ann. Trop. Med. Parasit., 1960, **54:**118–128.
71. Jung, R. C., and Beaver, P. C.: Clinical observations on *Trichocephalus trichiurus* (whipworm) in children. Pediatrics, 1952, **8:**548–577.
72. Kagan, I. G., et al.: Studies on the serology of visceral larval migrans. I. Hemagglutination and flocculation tests with purified Ascaris antigens. J. Immunol., 1959, **83:**297–301.
73. Kagan, I. G.: Trichinosis: A review of biologic, serologic and immunologic aspects. J. Infect. Dis., 1960, **107:**65–93.
74. Kagan, I. G.: Contributions to the immunology and serology of schistosomiasis. In Symposium: Resistance and immunity in parasitic infections. The Rice Institute Pamphlet, 1958, **45:**151–183.
75. Kagan, I. G., and Norman, L.: Immunologic studies on *Trypanosoma cruzi.* III. Duration of acquired immunity in mice initially infected with a North American strain of *T. cruzi.* J. Infect. Dis., 1961, **108:**213–217.
76. Kudo, R. R.: Protozoology. Thomas, Springfield, Illinois. 1954.
77. Lamont, N. McE., and Pooler, N. R.: Hepatic amebiasis—a study of 250 cases. Quart. J. Med. (N.S.), 1958, **27:**389–412.
78. Levine, N. D.: Protozoan Parasites of Domestic Animals and Man. Burgess, Minneapolis. 1961.
79. Levine, N. D.: Problems in the systematics of the "sporozoa." J. Protozool., 1961, **8:**442–451.
80. Lewert, R. M.: Invasiveness of helminth larvae. In Symposium: Resistance and immunity in parasitic infections. The Rice Institute Pamphlet, 1958, **45:**97–133.
81. Loran, M. R., et al.: Dependence of *Entamoeba histolytica* upon associated streptobacillus for metabolism of glucose. Exptl. Cell Res., 1956, **10:**241–245.
82. Maddison, S. E., Powell, S. J., and Elsdon-Dew, R.: Bacterial infection of amebic liver abscess. Med. Proc. Johannesburg, 1959, **5:** 514–515.
83. Manson-Bahr, P. E. C.: East African kala-azar, with special reference to the pathology, prophylaxis and treatment. Trans. Roy. Soc. Trop. Med. Hyg., 1959, **53:**123–136.
84. Manson-Bahr, P. E. C.: Manson's Tropical Diseases. Williams and Wilkins, Baltimore. 1954.
85. Manwell, R. D.: Introduction to Protozoology. St. Martin's Press, New York. 1961.
86. Markell, E. K., and Voge, M.: Diagnostic Medical Parasitology. W. B. Saunders Co., Philadelphia. 1958.
87. McConnachie, E. W.: Studies on *Entamoeba invadens,* Rodhain 1934, in vitro and its relationship to some other species of amoeba. Parasitology, 1955, **45:**452–481.
88. McCoy, O. R., et al.: Epidemic Amebic Dysentery. National Institutes of Health, Bull. No. 116. 1936.
89. McMullen, D. B., and Harry, H. W.: Comments on the epidemiology and control of bilharziasis. Bull. W. H. O., 1958, **18:**1037–1047.
90. Meleney, H. E.: Some unsolved problems in amebiasis. Amer. J. Trop. Med. Hyg., 1957, **6:**487–498.
91. Micks, D. W.: Insecticide-resistance, a review of developments in 1958 and 1959. Bull. W. H. O., 1960, **22:**519–529.
92. Miller, M. J., Neel, J. V., and Livingstone, F. B.: Distribution of the parasites in the red cells of sickle-cell trait carriers infected with *Plasmodium falciparum.* Trans. Roy. Soc. Trop. Med. Hyg., 1956, **50:**294–296.
93. Moore, D. V., and Connell, F. H.: Additional records of *Dipylidium canium* infections in children in the United States with observations on treatment. Amer. J. Trop. Med. Hyg., 1960, **9:**604–605.
94. Mueller, J. F.: Studies in *Sparganum mansonoides* and *Sparganum proliferum.* Amer. J. Trop. Med., 1938, **18:**303–328.
95. Neal, R. A.: Strain variation in *Entamoeba histolytica.* Parasitology, 1956, **46:**173–191.
96. Neal, R. A.: Enzymic proteolysis by *Entamoeba histolytica;* biochemical characteristics and relationship with invasiveness. Parasitology, 1960, **50:**531–550.
97. Nichols, R. L.: The etiology of visceral larval migrans. I. Diagnostic morphology of infective second stage Toxocara larvae. J. Parasit., 1956, **42:**349–362.
98. Offutt, A. C., Poole, B. A., and Fassnocht, G.

G.: A water-borne outbreak of amebiasis. Amer. J. Pub. Health, 1955, **45**:486–491.
99. Perkins, E. S.: Uveitis and Toxoplasmosis. Churchill, London. 1961.
100. Pesigan, T. P., et al.: Studies on *Schistosoma japonicum* infection in the Philippines. Bull. W. H. O., 1958, **18**:345–455; 481–578; **19**:223–261.
101. Phillips, B. P., et al.: Studies on the amebabacteria relationship in amebiasis. I. Comparative results of the intracecal inoculation of germ-free, monocontaminated, and conventional guinea pigs with *Entamoeba histolytica*. Amer. J. Trop. Med. Hyg., 1955, **4**: 675–692. II. Some concepts on the etiology of the disease. *Ibid.*, 1958, **7**:392–399.
102. Pizzi, T., and Taliaferro, W. H.: A comparative study of protein and nucleic acid synthesis in different species of trypanosomes. J. Infect. Dis., 1960, **107**:100–107.
103. Radke, M. G., et al.: Demonstrated control of *Australorbis glabratus* by *Marisa cornuarietis* under field conditions in Puerto Rico. Amer. J. Trop. Med. Hyg., 1961, **10**:370–373.
104. Report: Chagas' disease. W. H. O. Technical Report, Series No. 202. World Health Organization, Palais des Nations, Geneva. 1960.
105. Report: International Cooperation Administration Expert Panel on Malaria. Report and recommendations on malaria: a summary. Amer. J. Trop. Med. Hyg., 1961, **10**:451–502.
106. Report: International Work in Bilharziasis, 1948–1958. W. H. O. Chronicle, 1959, **1**:1–56.
107. Report: Onchocerciasis, Epidemiology, Pathogenesis, Chemotherapy, Vectors and Control. Bull. W. H. O., 1957, **16**:481–552.
108. Report: Symposium on laboratory aspects of amebiasis. Trans. Roy. Soc. Trop. Med. Hyg., 1957, **51**:303–331.
109. Rosen, L., et al.: Eosinophilic meningoencephalitis caused by a metastrongyloid lungworm of rats. J. Amer. Med. Assn., 1962, **179**:620–624.
110. Russell, P. F.: World wide malaria distribution, prevalence and control. Amer. J. Trop. Med. Hyg., 1956, **5**:937–994.
111. Sabin, A. H., et al.: Toxoplasmosis: current status and unsolved problems. Amer. J. Trop. Med. Hyg., 1953, **2**:360–444.
112. Sadun, E. H.: Studies on *Opisthorchis viverrini* in Thailand. Amer. J. Hyg., 1955, **62**:81–115.
113. Sadun, E. H., et al.: The detection of antibodies to infection with the nematode *Toxocara canis*, a causative agent of visceral larval migrans. Amer. J. Trop. Med. Hyg., 1957, **6**:562–568.
114. Sadun, E. H., Anderson, R. I., and Williams, J. S.: Fluorescent antibody test for the laboratory diagnosis of schistosomiasis in humans by using dried blood smears on filter paper. Exptl. Parasit., 1961, **11**:117–120.
115. Sasser, J. N., and Jenkins, W. R.: Nematology. University of North Carolina Press, 1960.
116. Schmidt, L. H., et al.: The transmission of *Plasmodium cynomolgi* to man. Amer. J. Trop. Med. Hyg., 1961, **10**:679–688.
117. Scott, H. H.: A History of Tropical Medicine. Williams and Wilkins, Baltimore. 1959.
118. Shaffer, J. G., and Ansfield, J.: The effect of rabbit antisera on the ability of *Entamoeba histolytica* to phagocytose red blood cells. Amer. J. Trop. Med. Hyg , 1956, **5**:53–61.
119. Shattuck, G. C.: Diseases of the Tropics. Appleton-Century-Crofts, New York. 1949.
120. Spencer, F. M., and Monroe, L. S.: The Color Atlas of Intestinal Parasites. Thomas, Springfield, Illinois. 1961.
121. Stirewalt, M. A., and Kruidenier, F. J.: Activity of the acetabular secretory apparatus of cercariae of *Schistosoma mansoni* under experimental conditions. Exptl. Parasit., 1961, **11**:191–211.
122 Stoll, N. R.: The worms: can we vaccinate against them? Amer. J. Trop. Med. Hyg., 1961, **10**:293–303.
123. Swerdlow, M. A., and Burrows, R. B.: *Dientamoeba fragilis*, an intestinal pathogen. J. Amer. Med. Assn., 1955, **158**:176–178.
124. Taliaferro, W. H., and Pizzi, T.: The inhibition of nucleic acid and protein synthesis in *Trypanosoma lewisi* by the antibody ablastic. Proc. Nat. Acad. Sci., 1960, **46**:733–745.
125. Taliaferro, W. H.: The inhibition of reproduction of parasites by immune factors. Bact. Rev., 1948, **12**:1–17.
126. Timms, A. R.: Schistosome enzymes. In Stauber, L. (ed.): Host Influence on Parasite Physiology, pp. 41–49. Rutgers University Press, 1960.
127. Trager, W.: Studies on the cultivation of malaria parasites. In Cole, W. H. (ed.): Some Physiological Aspects and Consequences of Parasitism, pp. 2–14. Rutgers University Press, 1955.
128. Trager, W.: Tsetse-fly culture and the development of trypanosomes to the infective stage. Ann. Trop. Med. Parasit., 1959, **53**:473–491.
129. Trussell, R. E.: *Trichomonas vaginalis* and Trichomoniasis. Thomas, Springfield, Illinois. 1947.
130. Vandepitte, J., and Delaisse, J.: Sicklémie et paludisme: aperçu du problème et contribution personelle. Ann. Soc. Belg Med. Trop., 1957, **37**:703–735.
131. Villella, J. B., Gomberg, H. J., and Gould, S. E.: Immunization to *Schistosoma mansoni* in mice inoculated with radiated cercariae, Science, 1961, **134**:1073–1075.
132. Vogel, H.: Acquired immunity to Schistosoma infection in experimental animals. Bull. W.H.O., 1958, **18**:1097–1103.
133. Vogel, H.: Über den *Echinococcus multilocularis* Süddeutschlands. Zeitschr. f. Tropenmed. u. Parasitol., 1957, **8**:404–456.
134. Von Bonsdorff, B.: *Diphyllobothrium latum* as a cause of pernicious anemia. Exptl. Parasit., 1956, **5**:207–230.
135. Von Brand, T.: Chemical Physiology of Endoparasitic Animals. Academic Press, New York. 1952.
136. Walton, B. C., et al.: The isolation and identification of *Trypanosoma cruzi* from raccoons in Maryland. Amer. J. Trop. Med. Hyg., 1958, **7**:603–610.
137. Wardle, R. A., and McLeod, J. A.: The Zoology of Tapeworms. University of Minnesota Press, 1952.
138. Weinman, D.: Toxoplasma and toxoplasmosis. Ann. Rev. Microbiol., 1952, **6**:281–298.
139. Weinstein, P. P., and Jones, M. F.: Development in vitro of some parasitic nematodes of vertebrates. Ann. N. Y. Acad. Sci., 1959, **77**: 137–162.
140. Sykoff, D. E., and Lepes, T. J.: Studies on

Clonorchis sinensis. I. Observations on the route of migration in the definitive host. Amer. J. Trop. Med. Hyg., 1957, **6**:1061–1065.

141. Yokogawa, M., et al.: Chemotherapy of paragonimiasis with Bithionol. Japanese J. Parasit., 1961, **10**:162–187.
142. Yokogawa, S., Cort, W. W., and Yokogawa, M.: Paragonimus and paragonimiasis. Exptl. Parasit., 1960, **10**:81–205.
143. Young, M. D., and Burgess, R. W.: Pyrimethamine resistance in *Plasmodium vivax* and *P. falciparum*. Bull. W. H. O., 1959, **20**:27–46.
144. Young, M. D., and Moore, D. V.: Chloroquin resistance in *Plasmodium falciparum*. Amer. J. Trop. Med. Hyg., 1961, **10**:317–320.

Chapter 35

RICKETTSIA

THE NAME rickettsia is applied to a group of very small, gram-negative coccobacillary microorganisms which includes the etiological agents of the typhus fevers, spotted fever and related diseases, and tsutsugamushi disease of the Far East.[6, 48, 67] They were first observed by Ricketts in 1909 associated with Rocky Mountain spotted fever. Those found in the bodies of lice taken from typhus fever patients by da Rocha Lima in 1916 were named by him *Rickettsia prowazeki* in honor of Ricketts who had died in 1910 of typhus fever during an investigation of that disease, and of von Prowazek, another early worker in the field who died of typhus. Closely similar microorganisms are found as the cause of heartwater disease in cattle, and of other diseases of lower animals, including sheep and dogs, and also, as apparently nonpathogenic forms, in the sheep ked, *Melophagus ovinus*.

Relation to Bacteria and Viruses. The rickettsiae are of special interest, not only as the causative agents of human disease, but because they appear to represent transitional forms between the bacteria on the one hand, and the viruses on the other. As described elsewhere (Chap. 9), the bacteria may be arranged in a series, from the purely saprophytic nonpathogenic forms through those which are saprophytic but pathogenic because they form potent toxins, to the closely adapted parasites which make up the majority of the disease-producing bacteria. Many of the last have fastidious nutritive requirements, reflecting limitations in synthetic abilities, and certain forms, especially *Pasteurella tularensis* and Bartonella, grow within the host cells, but all of these, with exceptions such as the leprosy bacillus and the spirochetes of syphilis and related diseases, are cultivable on lifeless mediums.

The rickettsiae, like the viruses, are set apart from the bacteria by their inability to grow in the absence of the living host cell, necessitating culture in the embryonated egg or in some other form of tissue culture. While it is a reasonable inference that they are dependent upon the host cell for essential metabolic reactions rather than essential metabolites, the rickettsiae do show limited metabolic activity and resemble the bacteria in having a similar cell structure and multiplying by a process of binary fission. They are related to the viruses through the viruses of the psittacosis group for which there is evidence for the occurrence of fission during replication, but which contain only fragments of enzyme systems, *viz.*, cytochrome C and folic acid. It is perhaps relevant that infections with rickettsiae and the psittacosis viruses are susceptible to treatment with the antimicrobial chemotherapeutic drugs, while the remainder of the diseases of viral etiology are not.

Although fragments of enzyme systems, such as flavin and biotin, are associated with certain of the pox viruses, and adenosine triphosphatase

activity is apparently an integral part of one of the avian leucosis viruses, most other viruses show little or no evidence of independent metabolic activity. Also, there is no evidence that any of these replicate by a process similar to fission. Rather, the viral particle seems to develop by differentiation within a morphologically discrete portion, or matrix, of the protoplasm of the host cell as in the case of the pox and related viruses, or final assembly seems to occur just prior to extrusion of the complete virus particle from the host cell as in the replication of influenza virus (Chap. 4).

The rickettsiae, then, although occupying an intermediate position, seem to be more nearly related to the bacteria than to the viruses, but whether such a graded series of microorganisms has phylogenetic implications is another matter (Chap. 7).

Morphology.[77] These microorganisms may appear either as cocci or short bacilli, 0.3 to 0.5 μ long and 0.3 μ in breadth, but bacillary forms as long as 2 μ may occur, and filamentous forms have been described. The cells occur singly, in pairs perhaps to be associated with binary fission, and often in dense irregular masses, within affected cells, particularly those of mesothelial origin which line the serous cavities. Some species are found only in the cytoplasm, but others, such as those of the spotted fever group, occur in the nucleus as well. They are nonmotile and do not show obvious capsules.

In electron micrographs of isolated rickettsiae prepared in the usual way there appears to be a dense central portion surrounded by a peripheral area of less dense material, but when the preparation is fixed with osmic acid, or is a section of infected tissue, the dense central portion is not differentiated. A well-defined cell wall is present and demonstrable in electron micrographs.

Staining. The rickettsiae stain poorly or not at all with the usual bacterial stains but are readily stained with Giemsa, are stained red against a blue background with Macchiavello's stain (basic fuchsin, citric acid, and methylene blue), and a light blue against a pink background with Castaneda's stain (buffered methylene blue and safranine counterstain). The coccoid and coccobacillary forms stain uniformly, but bipolar staining of the bacillary forms in not uncommon.

Chemical composition. The chemical composition of typhus and Q fever rickettsiae is closely similar to that of most bacteria. There is some uncertainty as to the precise amounts of the various components present because of possible, or

Figure 254. The rickettsiae of tsutsugamushi disease in scrapings from a mesothelial surface of an infected guinea pig. Note the coccobacillary forms, often paired, and their intracellular location. × 2000.

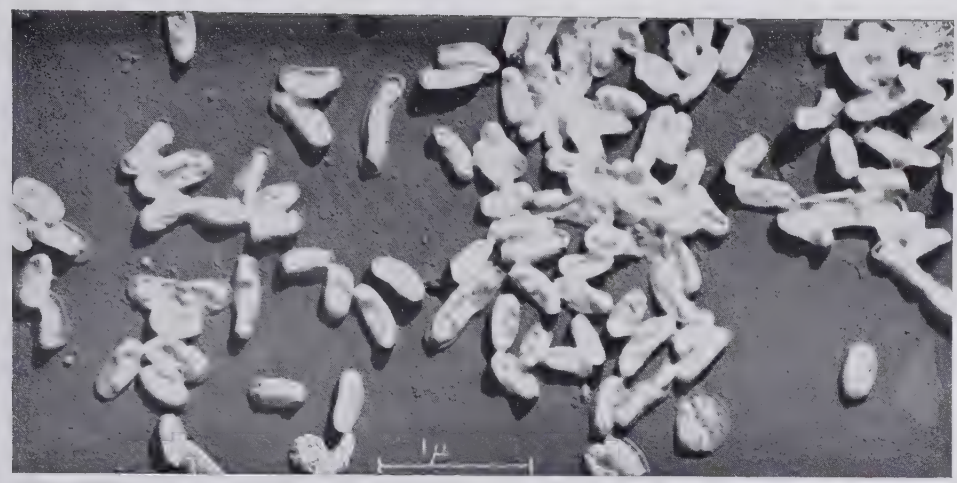


Figure 255. Electron micrograph of Q fever rickettsiae purified by elution from a cellulose anion exchanger. (Hoyer et al.[28])

even probable, loss during purification procedures such as, for example, ether extraction and washing. Preparations not treated with ether have given about 30 per cent protein, 47 per cent lipid including 30 per cent neutral fat and 17 per cent phospholipid, and 12 per cent nucleic acid. The lipid figure is in contrast with that of 12 to 13 per cent found in ether-extracted and washed preparations, but it is uncertain as to whether the differential represents contamination or rickettsial lipid removed by the extraction. The nucleic acid fraction contains both the deoxypentose and pentose types, in a ratio of about 2:1, with relatively constant amounts of DNA, but variable amounts of RNA from one preparation to another. Cell wall preparations from rickettsiae lysed with desoxycholate resemble bacterial cell walls in composition, containing amino acids and a polysaccharide moiety hydrolyzing to glucose, galactose, hexosamine, and glucuronic acid.[56]

Physiology.[15] The occurrence of independent metabolic reactions of rickettsiae has been noted above. Largely through the work of Bovarnick, Wisseman, and others,[10, 27] it has become clear that these microorganisms carry out glutamic-aspartic acid transamination and oxidize glutamic acid via the citric acid cycle. It has been found also that glutamate oxidation can lead to the phosphorylation of hexose and the formation of adenosine triphosphate from the diphosphate.[50] The formation of glucose-6-phosphate is catalyzed by a hexokinase which is similar to hexokinase from other sources.[45] A terminal respiration mechanism is indicated by the presence of flavin-iron-porphyrin catalysts,[26] but the utilization of glucose has not been shown. Folic acid is present and a possible function is suggested by the synthesis of serine from glycine and formaldehyde in the presence of tetrahydrofolic acid[44] by the Q fever rickettsia.

These activities are not stable and the conditions effecting their inactivation and reactivation are of considerable significance. On storage in isotonic salt solution in the cold, rickettsiae lose the ability to carry out the above reactions and at the same time lose toxic and hemolytic activity (see below) and infectivity. It has also been known from early work on spotted fever by Parker and others that the rickettsiae in starved ticks are avirulent but become virulent after the tick is fed or incubated at body temperature. Loss of toxic and hemolytic activity also occurs at 36° C. in the absence of metabolizable substrate.

In the first two instances, physiological activity, toxic and hemolytic activity, and infectivity are restored by incubation of the microorganisms with DPN and/or coenzyme A.[11, 12] Para-amino-

benzoic acid has a rickettsiostatic effect (see below), and this inhibitory effect is also reversed by DPN.[25] The inhibitory effect of PABA is thought to be due to the formation of a PABA-DPN complex, thus preventing dehydrogenase activity, and reactivation occurs when exogenous DPN is added to the system.

Inactivation of toxic and hemolytic activity at or near body temperature is possibly different. It is prevented in the presence of glutamate, pyruvate, or ATP, and reactivation occurs on incubation with glutamate and inorganic phosphate and is increased by ATP, the gain or loss of activity being correlated with ATP. Evidence such as the foregoing provides a rational basis for the nature of the suspending medium, a sucrose-phosphate-glutamate solution, used for rickettsiae.

Cultivation. As indicated above, the rickettsiae grow only in the presence of, and probably invariably within, host cells. They will grow on the chorioallantoic membrane of the embryonated hen's egg, but usually produce only small numbers. As first shown by Cox, they grow readily and profusely in the yolk sac of the embryonated egg, and this method of culture has been one of the most widely used.

They also grow in various kinds of tissue culture. In modified Maitland medium, proliferation of the rickettsiae is not associated with active cell metabolism, as is usually the case with the viruses, but rather with a low metabolic rate of the host tissue, and proliferation is most rapid as the host cells begin to die. Active multiplication has, in fact, been observed in dead chick embryos which contain some surviving cells.

Several species of rickettsiae have been studied in cultures of mouse fibroblast tissue culture, using a cell line (MBIII) that grows in roller tubes as a dispersed suspension of individual cells.[13] The rate of growth was relatively slow, with a generation time of about 16 hours, which compares with the short generation time of rapidly multiplying bacteria of 20 minutes or less, and the longer generation time of 30 hours or more of slow-growing bacteria such as tubercle bacilli. Studies of growth in rat fibroblast culture in the living state by phase microscopy have made possible the direct observation of the occurrence of binary fission.[57] It was also observed that, following intracellular multiplication, the microorganisms are extruded from the host cells in association with a process of extension and retraction of microfibrils at the surface of the cell. It is probable, however, that rickettsiae are usually released by degeneration and dissolution of the host cell. Cultures of explants of the endodermal cells of the chick yolk sac have also been used for

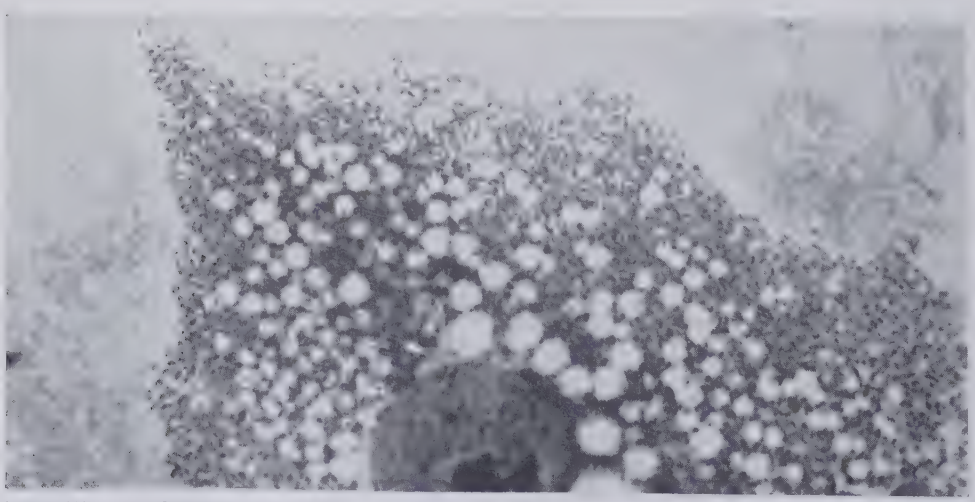


Figure 256. *Rickettsia prowazeki* in an infected cell from chick endoderm cultured *in vitro*. Most of the cytoplasm of the cell is occupied by rickettsiae. × 1500. (Weiss.)

the cultivation of rickettsiae[69, 73] and may be used for quantitative titration of infection, at least with the Q fever rickettsiae, because foci of infected cells are readily observed in stained preparations and may be counted.

Resistance. The resistance of the rickettsiae to deleterious influences is generally of the same order as that of the more delicate bacterial vegetative cells. They are killed by the usual disinfectants but, like some of the viruses, are resistant to glycerol, and by heating at 55 to 60° C. for 30 to 60 minutes. The Q fever rickettsiae are said to be somewhat more resistant to heating, and these and the typhus fever rickettsiae are relatively resistant to drying, persisting in viable infectious form in the airborne state in the first instance, and in infected louse feces in the second, for considerable periods.

Pathogenicity.[60] The rickettsiae appear to be well-established parasites of arthropods, and there is good evidence for the acarinid origin of the rickettsial diseases. They are not pathogenic for the insect vectors that transmit them, except lice, and, in fact, the infection is congenital in insects undergoing an incomplete metamorphosis, *e.g.*, ticks. They also appear to be reasonably well adapted to certain animals, especially rodents, which in many instances constitute a reservoir of infection in nature.

The characteristic pathological changes found in rickettsial disease result from the multiplication of the microorganisms within the endothelial cells of the small blood vessels throughout the body, and especially in the skin and brain in the case of typhus fever. Proliferation of affected cells, and thrombosis following injury to the lining of small vessels, is common. Inflammatory cells accumulate around areas of vessels, and in severe infections in man and guinea pigs, *e.g.*, those of spotted fever, the smooth muscle cells of the vessel media may be involved, and extensive thrombosis can lead to gangrene.

Toxin. Two kinds of rickettsial toxicity are demonstrable, the one an endotoxin associated with the intact microorganisms[7, 16] and the other a hemolytic activity which lyses sheep and rabbit cells but not those of a number of other species examined. The endotoxin is lethal for mice and is titrated by intravenous inoculation of material such as yolk sac culture, with deaths up to 18 hours attributable to toxicity. The titer of the toxin is directly proportional to the number of rickettsiae present and is correlated with their viability. Most agents that destroy infectivity also inactivate the toxicity, with the exception of ultra violet radiation which, in appropriate doses, selectively reduces infectivity without affecting toxic, hemolytic, or respiratory activity.[2] The activity of the toxin is similar to that of other microbial endotoxins and increases vascular permeability but appears to differ in other respects.[76] It is specifically neutralized by antiserum, with cross-reactions between members of the main groups of rickettsiae, the typhus, spotted fever, and tsutsugamushi groups (see below), but not between groups. There is also an exotoxic activity demonstrable by intradermal inoculation of the rabbit to produce a local inflammatory reaction and by enlargement of the liver and spleen of rats and mice on intraperitoneal inoculation.

The hemolytic activity of suspensions of purified rickettsiae is species-specific, as noted above, and is closely associated with both endotoxin and enzymatic activity, particularly the oxidation of glutamate, and its activity is inhibited by metabolic inhibitors. Ordinarily hemolytic activity of the causative microorganism does not play an appreciable part in the pathogenesis of infectious disease, but there are certain exceptions such as *Clostridium welchii* infections. It is concerned in fatal rickettsial toxemia in the rabbit also, the animals showing massive intravascular hemolysis, with hemoglobinemia, hyperkalemia, myocardial damage, and hypotension. It is not clear, however, that either the toxicity or hemolytic activity plays any significant part in the patho-

genesis of rickettsial infections, for they appear to be dissociable from virulence.[49]

Experimental infections. Rickettsial infection may be produced in the guinea pig, the usual procedure in isolation being the intraperitoneal inoculation of 5 ml. of blood into each of two animals. A febrile attack occurs in 7 to 12 days, and some varieties of rickettsiae produce a characteristic orchitis or scrotal reaction which has differential value. An acute fibrinous exudate is formed in the scrotal sac, and many cells packed with rickettsiae may be found. The disease in the guinea pig is often not fatal, and this animal is much more resistant to infection than is man. Other common laboratory animals are more resistant, and rats, mice, rabbits, dogs, cats, etc., may show no febrile reaction or other outward manifestation of disease, though the infection has been established and in some cases, at least, may persist for months.

Chemotherapy. Sulfonamides and penicillin are ineffective in the chemotherapy of the rickettsial diseases, although they have been used in the past in the treatment of complications such as bronchopneumonia and other secondary bacterial infections. Para-aminobenzoic acid has been found to be partially effective in typhus and spotted fevers if given early, but cannot be regarded as a really effective chemotherapeutic agent. With the development of the broad spectrum antibiotics effective chemotherapy of the rickettsial infections has been made possible, for these substances have been found to be effective chemotherapeutic agents for this group of diseases. Chloramphenicol has been of particular interest in the treatment of tsutsugamushi disease (scrub typhus), since effective prophylactic immunization of man is not yet practical and the mite reservoir of infection is not subject to adequate control. Largely through the work of Smadel and his co-workers[59] it has been found that the clinical disease may be suppressed by prophylactic or therapeutic administration of this substance but that relapse occurs unless the treatment is continued for about 4 weeks; apparently the antibiotic alone does not suffice to eliminate rickettsiae from the tissues, but an immune response is required also. The tetracyclines have been found to be even more effective chemotherapeutic agents.

It has been of considerable interest that, although drug-resistant strains of rickettsiae have not yet been found in nature, Weiss and his colleagues have been able to develop resistant strains in the laboratory. Strains of *R. prowazeki* resistant to the rickettsiostatic activity of PABA, quinoxaline and erythromycin have been produced by serial egg passage in the presence of increasing concentrations of the drug.[70, 71, 72] Most, but

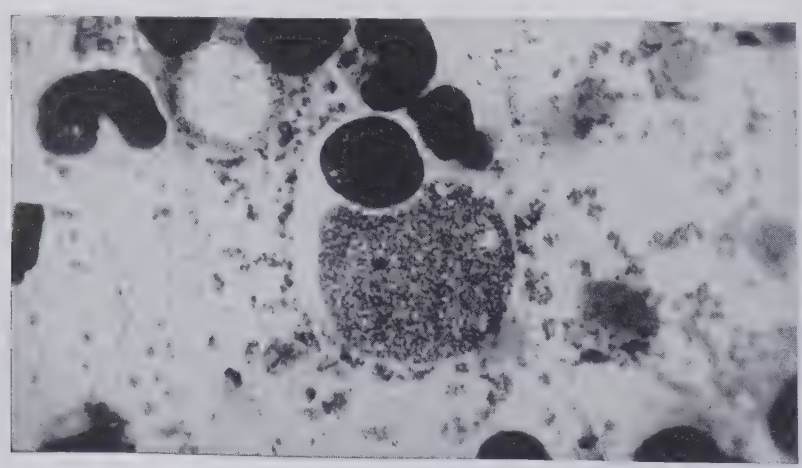


Figure 257. Rickettsiae of Q fever in a smear of skin exudate. Note the macrophage packed with enormous numbers of the rickettsiae. × 1125.

not all, of the resistant strains did not appear to be altered in virulence and toxicity.

Immunity. Recovery from an attack of rickettsial disease usually confers a solid and lasting immunity. In at least some instances this is associated with the prolonged survival of the microorganisms in the body as indicated by the occurrence of Brill's disease (see below) and the demonstration of tsutsugamushi rickettsiae in clinically recovered individuals.

The development of artificial immunization procedures will be considered later, but it may be noted here that the immune response is manifested by the appearance of protective antibody, of opsonins and agglutinins for the rickettsiae, and of complement-fixing antibody. The last has been of a good deal of interest as a differential diagnostic method and distinguishes even closely related varieties of rickettsiae. Antibody neutralizing the toxicity of typhus rickettsiae occurs also and is used in the United States as the basis of assay of immunogenic potency of vaccines. The rickettsiae contain both somatic and capsular antigens. In the purification of yolk sac culture the material is extracted with ether to remove lipid material, and, with the exception of *R. orientalis*, the rickettsiae are found in the aqueous phase. The extraction results in the liberation of soluble, group-specific antigen in the aqueous phase which has been found to be stripped-off capsular material.

The Weil-Felix reaction. In some rickettsial diseases agglutinins appear to certain strains of Proteus. This apparently anomalous response was observed by Weil and Felix in 1915 and has very considerable diagnostic value. They found that a strain of *Proteus vulgaris*, which they designated X-2, was agglutinated by typhus serums, and another strain, designated X-19, was similarly agglutinated but to much higher titer; in fact, titers as high as 1:50,000 are occasionally observed in European typhus. It was further shown that the agglutinating antigen was a part of the heat-stable somatic or O antigen; these strains are, therefore, commonly termed OX strains. Other strains showing this immunological characteristic have been isolated since. It is of some importance that an O antigen be used in carrying out the agglutination because antibody to the flagellar antigen of Proteus occurs with some frequency in normal serums. More general application of this agglutination test, commonly known as the Weil-Felix reaction, showed that it is specific for the typhus group of fevers, serums from other rickettsial diseases agglutinating only to low titer or not at all. The phenomenon is a consequence of the presence of a common antigen in the typhus rickettsiae and the X strains of Proteus.

The Proteus agglutinin is only a part

Immunological (Weil-Felix) Grouping of Rickettsial Diseases*

IMMUNOLOGICAL GROUP		
OX-19	OX-K	Undetermined
OX-19 ++++	OX-19 −	OX-19 +
OX-2 +	OX-2 −	OX-2 +
OX-K −	OX-K ++++	OX-K +
Classic, European typhus	Tsutsugamushi of Japan, Formosa, Malay, Netherlands East Indies	Spotted fever
Brill's disease	"Scrub typhus" or "rural typhus" of Malay, N.E.I., French Indo-China, India, Australia	São Paulo typhus
Endemic, murine typhus of United States, Australia, Greece, Syria, Manchuria, Malay (shop typhus), India, Burma, Philippines, Hawaii, Toulon (fièvre nautique)	Mite fever of Sumatra	Fièvre boutonneuse
		Fièvre exanthematique
		Febbre errutiva
		Tick fever of South Africa
		Kenya fever
		Tick typhus of India

* Modified from Felix.

Rickettsial Diseases of Man

	NAME OF RICKETTSIA	SYNONYMS	DISEASE	GEOGRAPHIC DISTRIBUTION	ARTHROPOD VECTORS	VERTEBRATE RESERVOIR	EXPERIMENTAL INFECTIONS AND OBSERVATIONS	WEIL-FELIX REACTION	COMPLEMENT-FIXATION
Typhus group	*Rickettsia prowazeki*	.....	Epidemic typhus (classic, European, louse-borne typhus) Brill's disease	Europe, Asia, S. America N. America, Europe, etc.	Human louse, *Pediculus humanus*	Man	G. pig; fever only	OX-19: ++++ OX-2: + OX-K: 0	Group- or species-specific
	Rickettsia typhi	*Rickettsia mooseri* *R. murícola*	Murine typhus, (endemic typhus, flea-borne typhus)	World-wide	Fleas *Xenopsylla cheopis* *X. astia* Rat louse *Polyplax spinulosa*	Wild rats Field mice	G. pig; fever and scrotal swelling	OX-19: ++++ OX-2: + OX-K: 0	
Spotted fever group	*Rickettsia ricketitii*	*Dermacentroxenus rickettsi*	Spotted fever (Rocky Mt. spotted fever) São Paulo typhus Tobia fever	North America Brazil Colombia	Ticks *Dermacentor andersoni* *D. variabilis* *D. parumapertus* *Rhipicephalus sanguineus* *Haemaphysalis leporis-palustris* Several species of *Amblyomma* *Ixodes dentatus*	Incomplete evidence indicates: rabbits, small rodents, dog, opossum (S. America)	G. pig; fever and severe scrotal reaction (necrosis)	OX-19: + OX-2: + OX-K: 0	
	Rickettsia conori	*Dermacentroxenus rickettsi* *R. rickettsii* var. *conori* *Dermacentroxenus sibericus?*	Boutonneuse fever (Marseilles or Mediterranean fever) Indian tick typhus? Kenya typhus? South African tick-bite fever? Siberian tick typhus?	Mediterranean littoral India East Africa South Africa Central Asia	Ticks *Rhipicephalus sanguineus* *R. appendiculatus* *Haemaphysalis leachii* *Amblyomma hebraeum*	Dog Rodents? (S. Africa)	G. pig; fever and scrotal swelling	OX-19: + OX-2: + OX-K: 0	Group- or species-specific
	Rickettsia australis	.....	North Queensland tick typhus	Australia	Tick? *Ixodes holocyclus?*	Rat? Marsupials?	G. pig; fever and scrotal swelling	OX-19: + OX-2: + OX-K: 0	
	Rickettsia akari	.....	Rickettsialpox	Urban localities in N.E. United States and in USSR	Mite *Allodermanyssus sanguineus*	House mice	Mouse; ascites, splenomegaly, death	OX-19: 0 or + OX-2: 0 OX-K: 0	

Tsutsugamushi group	*Rickettsia tsutsugamushi*	*Rickettsia orientalis* *R. nipponica* *R. akamushi*	Tsutsugamushi disease Japanese river fever Kedani fever Scrub typhus Rural typhus	Japan, Korea, China, Philippines, S.E. Asia, India, Indonesia, N. Australia	Mites *Trombicula akamushi* *T. deliensis* *T. pallida*	*Apodemus agrarius* and other wild rodents Birds?	Mouse; ascites, splenomegaly, death	OX-19: 0 OX-2: 0 OX-K: ++++	Not yet adequately studied
	Coxiella burnetii	*Rickettsia burnetii* *R. diaporica*	Q fever	World-wide	Ticks *Haemaphysalis humerosa* *Ixodes holocyclus* *Dermacentor andersoni* *Amblyomma americanum* (and isolations from many others)	Bandicoot (Australia) Birds? Human infections occur from direct or indirect contact with infected sheep, cattle, goats	G. pig; fever only	OX-19: 0 OX-2: 0 OX-K: 0	Specific
	Rickettsia quintana	*Rickettsia pediculi* *R. wolhynica*	Trench fever (Wolhynian fever; 5-day fever)	Recognized only during World Wars I and II on European fronts	Human louse, *Pediculus humanus*	?	None	OX-19: 0 OX-2: 0 OX-K: 0	Not available

of the antibody response. It is not identical with the rickettsia agglutinin for the latter appears earlier, persists longer, and appears to be associated with protective antibody. Furthermore, the opsonin remains in typhus serum after it has been absorbed with Proteus.

Another immunological type of Proteus, the OX-K strains, was found to be agglutinated by tsutsugamushi serums but not by typhus serums. This, coupled with the OX-19 type, makes possible the division of the rickettsial diseases into three groups: the typhus group in which OX-19 is agglutinated, the tsutsugamushi group in which OX-K is agglutinated, and the spotted fever group which is indeterminate in that neither type of Proteus is agglutinated to high titer. A classification of the rickettsial diseases on this basis is given in the accompanying table.

The significance of the Weil-Felix reaction is wholly unknown. The occurrence of one common antigen, or more specifically a polysaccharide haptene, in two such different microorganisms might be a matter of chance. The occurrence of a second common antigen, shared by another variety of Proteus and another group of rickettsiae, on the basis of chance alone is improbable. There is no evidence, however, which points to any other connection between Proteus and the rickettsiae. There is, for example, no association between the X strains and typhus; X strains are found in diseases other than typhus, and Proteus strains other than X strains are found in typhus. Proteus infections, of course, often result in the formation of specific agglutinins. It has been found, for example, that the serums of persons infected with *Pr. vulgaris, Pr. mirabilis,* or *Pseudomonas pyocyanea* will agglutinate OX-K strains of Proteus to a high titer. Such persons might be said to give a positive Weil-Felix reaction.

Specific serological reactions. An accurate method of serological diagnosis and identification should be based on the use of rickettsial antigen and thus a true immunological specificity. Although it has been extremely useful, the Weil-Felix reaction is not a specific reaction and is not completely reliable. The protection test in the guinea pig is useful in the demonstration of antigens functional in effective immunity, but the relatively greater natural resistance of these animals as compared with that of man tends to blur differentiation in some instances. There is, for example, a significant and adequate cross-protection between louse-borne and murine varieties of typhus rickettsiae in the guinea pig, but not in more highly susceptible man. Similarly, the protection test does not differentiate between strains of Q fever rickettsiae.

The *in vitro* serological reactions, made practicable by the availability of relatively large amounts of antigen from yolk sac culture, are of two kinds, the one direct agglutination of rickettsiae in suspension, and the other complement fixation. Two kinds of antigen are present, group-specific soluble antigen and strain-specific antigen present in the microorganisms.

Yolk sac cultures contain large amounts of lipid material which is removed by ether extraction of a saline suspension of the yolk sac material. As indicated above, all the rickettsiae except the tsutsugamushi rickettsiae, which are found in the interphase emulsion, remain in the aqueous phase, and the treatment releases soluble antigen from all of these except Q fever rickettsiae. The soluble antigen can be separated by spinning out the rickettsiae in the centrifuge and used in a precipitin test, or the mixture of rickettsiae and soluble antigen can be used as an antigen in the complement-fixation test.

Difficulty has been experienced with some antigen preparations in that they tend to give false positive complement-fixation reactions, especially with Wassermann positive serums. This is not true of suspensions of washed rickettsiae, but these are difficult and laborious to prepare. Satisfactory soluble antigens may be prepared from infected yolk sacs purified by ether extraction, followed by treatment with benzene and precipitation with sodium sulfate. Complement

fixation with this antigen does not give false positives with Wassermann positive serums, but does not always distinguish between epidemic and murine typhus.

The fluorescent antibody technique has been applied to the demonstration of rickettsiae by the direct method,[14, 35] and to the titration of antibody by the indirect method. Troublesome cross reactions in the latter, as between murine and epidemic typhus, are largely eliminated by the simple expedient of diluting the serum to be tested in heterologous soluble antigen.[22]

Classification. The intermediate position of the rickettsiae with respect to other microorganisms has been described above, but it is also apparent that differentiation occurs within the group. In general, the rickettsiae pathogenic for man fall into four groups. Three of these are well defined with respect to immunological character and pathogenicity but are heterogeneous with respect to insect vectors. These are the rickettsiae responsible for the typhus group of fevers, those of the spotted fever group, and the causative agents of tsutsugamushi disease. There is some confusion as regards the names of diseases caused by these forms because a number of the spotted fever group of diseases have been given names such as "tick typhus." The rickettsiae causing Q fever and trench fever are set apart from the other rickettsiae, but are unrelated to one another, and make up a miscellaneous group.

Within the typhus group, two species are distinguished, *R. prowazeki* and *R. typhi,* the causative agents of epidemic louse borne or European typhus, and of murine typhus respectively. Four species are distinguished within the spotted fever group: *R. rickettsii,* the causative agent of spotted fever, São Paulo typhus, and Tobia fever; *R. conori,* the causative agent of Boutonneuse fever and the probable causal agent of tick-borne diseases occurring in Africa, India, and Siberia under the general name of tick typhus; *R. australis,* the causative agent of Australian tick typhus; and *R. akari,* the causative agent of rickettsialpox. The tsutsugamushi rickettsiae are immunologically heterogeneous, but subgroups or species have not been distinguished, and these agents are given the single name *R. tsutsugamushi.*

The apparent causative agent of trench fever is poorly known, and the designation *R. quintana* is not based upon a detailed differential characterization. The causative agent of Q fever has, however, been characterized with some precision and is set apart from the other rickettsiae sufficiently that it has been given a different name, *Coxiella burnetii.* The occurrence and differential characteristics of these rickettsiae are summarized in the accompanying table.

The rickettsiae found in lower animals are also thought to differ from the human pathogens sufficiently to warrant different names, *Cowdria ruminatum* for the causative agent of heartwater disease, and others causing disease of lower animals are designated Ehrlichia, including *Ehrlichia ovina* found in sheep, *E. canis* found in dogs, and *E. bovis* found in cattle. The rickettsia-like nonpathogenic microorganism is found in the sheep tick and, unlike the rickettsiae cultivable on glucose-blood agar, has been named *Wolbachia melophagi.*

THE TYPHUS FEVERS[36, 53]

The typhus fevers constitute a group of closely related infections which occur in various parts of the world. The incubation period is 5 to 18 days, and the clinical picture is essentially the same in all the typhus fevers, though the severity of the disease varies widely. There is an initial violent headache which persists with the onset of chills and high fever. A macular eruption appears soon after

the fourth day which remains until defervescence. Crisis occurs at about the twelfth day, and recovery may be more or less complete at the end of another 2 weeks, though the cough which develops may persist, and mental vigor may remain somewhat impaired for some time. The complications include typhus gangrene, which is perhaps associated with the circulatory disturbances arising during the disease, a highly fatal bronchopneumonia, otitis media, and typhus encephalitis. Death rarely occurs before the end of the first week. The case fatality is highly variable; it has been as great as 70 per cent in some epidemics, and 20 to 30 per cent is not uncommon, while in endemic typhus it is much lower, perhaps 5 per cent. Typhus is almost always a considerably milder disease in children than in adults.

Two species of rickettsiae are responsible for these diseases, and characteristically occur in the cytoplasm but not in the nucleus of invaded cells. They are associated with two epidemiologically different types of typhus fever. One is the classic European or epidemic typhus and the etiological agent is *R. prowazeki* (*R. prowazeki* var. *prowazeki*). The other is murine typhus, sometimes called endemic typhus, and the causative rickettsia is *R. typhi* (*R. prowazeki* var. *mooseri, R. muricola, R. mooseri*). Both are immunologically homogeneous, and strains from all parts of the world appear to be substantially identical. These rickettsiae may be distinguished from one another by complement fixation and contain both common and individual antigens.

EPIDEMIC LOUSE-BORNE TYPHUS FEVER

The classic form of typhus fever known for many years is the louse-borne typhus of Central Europe which persists in endemic foci in Russia and Poland and has occasionally broken out in major epidemic form from time to time during periods of stress. The disease is associated with overcrowding and filth and has been termed "camp fever" and "jail fever." Epidemics are not infrequent in both civil and military populations during time of war and may become very extensive; it is estimated, for example, that 315,000 persons died of typhus in Serbia in 1915 and that twenty-five million cases occurred in Russia in 1917 to 1921.

The disease is transmitted by the human body louse, *Pediculus vestimenti*. The head louse may also transmit the infection, but its importance in the spread of the disease is not established. During the febrile stage of the disease, rickettsiae are present in the blood, and the louse is infected by ingestion of blood. In the louse the rickettsiae infect the cells lining the gut, multiply, and are discharged in the feces after rupture of the infected cells, the louse becoming infective in 6 to 8 days at 32° C. The salivary glands are not infected, congenital transmission of the infection does not occur, and the louse is eventually killed by the infection.

Most human infections are probably acquired by contamination of the louse bite with infected feces, but infection via the mucous membranes is possible. The rickettsiae persist in viable form in dried material such as louse feces, and accidental laboratory infections have occurred following inhalation of infectious materials.

Although bedbugs and ticks have been experimentally infected, it is probable that the louse is the sole vector of the disease under natural conditions.

Brill's Disease. Louse-borne European typhus of a mild type is endemic, though in late years decreasing in prevalence, in cities along the Atlantic Coast in this country. Long regarded by many as different from European typhus, Brill's disease has been shown to be louse borne and identical with European typhus. In this country it has presumably been imported from endemic areas in Europe. It has also been found in Australia[66] in immigrants from Central Europe. Brill's disease, or latent typhus, has been observed in Europe, both in

England[33] and on the continent. Such latent typhus, or healthy carrier state, capable of infecting lice fed upon the infected individual, may provide the starting point of epidemics. Russian workers tend to discount such a source of infection, considering that apparent recurrence is a consequence of reinfection rather than relapse.[46] Since the infection is not congenitally transmitted in the louse, and infected lice usually die within 2 weeks, it seems probable that the reservoir of infection occurs in man.[51] Such a reservoir of subclinical infection in Egypt, for example, is indicated by serological surveys.[1] The reservoir known to exist in Central Europe may consist in part of latent infections, but the disease smolders in endemic form in relatively large numbers of mild infections. For example, an over-all incidence of 24.5 per cent of positive Weil-Felix reactions was reported in healthy Russians with no history of typhus in the German-occupied area of Russia in World War II, rising from 11 per cent in the less than 5 age group to 34.5 in the 6 to 10 age group.

MURINE TYPHUS

The type of typhus fever which prevails in the southern United States and in Mexico, where it is known as *tabardillo,* was long assumed to be a mild form of louse-borne European typhus. The disease is associated with rats, and it has since become apparent through the investigations of a number of workers that the rat is a reservoir of infection in both this country and Mexico and that the disease is transmitted from rat to rat and from rat to man by the rat flea, *Xenopsylla cheopis,* and by the rat louse, *Polyplax spinulosa.*

The disease may also be transmitted by the human louse and, when a case occurs by transmission from the rat in a community where there are lice in abundance, it has been suggested that murine typhus may become an epidemic louse-borne infection. Such epidemics occur from time to time in Mexico. Murine typhus occurs with considerable frequency in this country; approximately 20,000 cases were officially recorded from 1932 to 1941, and 5191 cases with 214 deaths were reported in 1945 from forty-four states but predominantly from the southeastern states. Prior to 1946 it was generally believed that murine typhus was an under-reported disease in this country. Since that year it has been apparently declining rapidly, with 685 cases reported in 1950 and 46 cases in 1961. With attention directed toward the disease, fallacious diagnosis appears to have become more common, largely as a result of misinterpretation of low-titer Weil-Felix reactions. With the advent of specific complement fixation, many reported cases are not confirmed by this method. In a study in the southeastern states it was shown, for example, that 25 per cent of reported cases were definitely not typhus.[54] A degree of control of the disease is possible by reduction of rat and rat flea populations, as indicated by a study in Georgia in which the incidence of human disease was reduced by 80 to 90 per cent.[38]

Murine typhus also occurs elsewhere in the world and is known by various names. The urban type occurring in Malaya or "shop typhus," the mild form of the disease in the Mediterranean region known as *Toulon typhus,* and other infections such as *Moscow typhus, Manchurian typhus,* and the *Red Fever of the Congo* are all murine typhus identical or nearly so with the type found in the United States and Mexico.

Clinically murine typhus does not differ appreciably from the classic European type. It is sometimes said that the European disease is likely to be more often fatal, but this does not appear to be true; both forms are equally fatal in epidemic form and relatively mild in the endemic form.

Differences between *R. prowazeki* and *R. typhi* are demonstrable but not great. The murine rickettsiae produce a necrotic scrotal reaction in guinea pigs, while the European variety does not; the murine variety may be carried indefi-

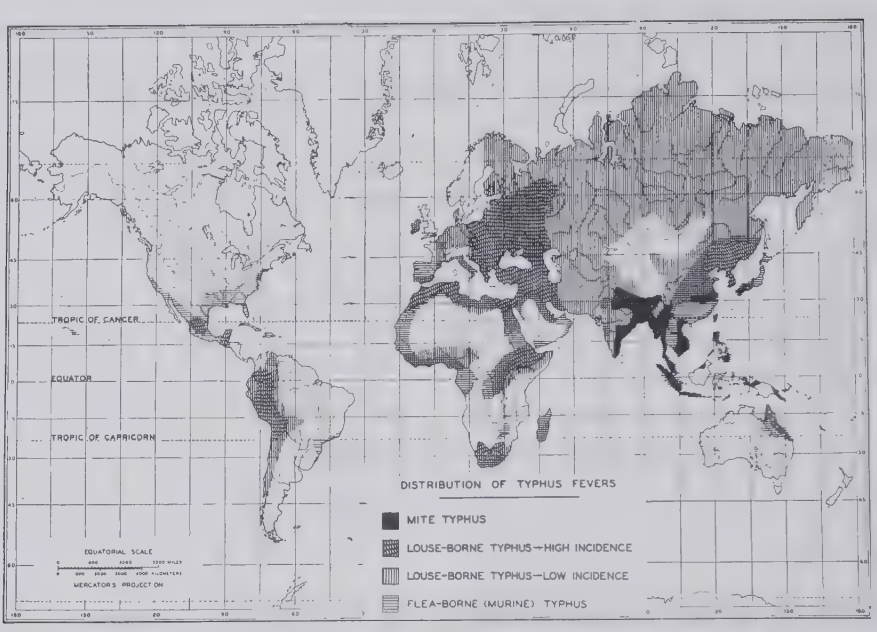


Figure 258. The world-wide distribution of typhus fevers, including classic or European typhus, murine typhus, and mite typhus of the Far East. Redrawn from maps prepared by Army Medical Intelligence. (Based on Goode *Base Map* No. 201M. By permission of the University of Chicago Press.)

nitely in mice without alteration, but the European variety tends to degenerate; the rickettsial pneumonia and intraperitoneal multiplication in rats with the production of enormous numbers of rickettsiae may be produced by the murine variety but not by the European type alone. There is a slight immunological difference also, for, while recovery from either infection results in a solid and lasting immunity to both, murine vaccines protect against murine infection but only incompletely against infection with the rickettsiae of European typhus. The two may be differentiated by the complement-fixation test.

IMMUNIZATION

The preparation of vaccines which will produce an effective active immunity to typhus fever is a matter of some importance, since under the circumstances in which it occurs in epidemic form adequate control of the vector is often not possible. In man the immunity produced by immunization is specific, and the cross-immunity between epidemic and murine typhus is not of a sufficiently high order to be practical.

Inactivated Vaccine. Rickettsiae have been grown in a number of ways for preparation of inactivated vaccines. Weigl vaccine was one of the first and was prepared from the bodies of lice infected by intrarectal inoculation. A vaccine prepared from the infected mouse brain has been used also. *R. typhi* produces a pneumonia in rats on intratracheal inoculation, and rickettsiae may be recovered in large numbers from infected lung tissue by differential centrifugation. The rat is not susceptible to this kind of infection with *R. prowazeki* unless its resistance is reduced by prior treatment such as x-irradiation. These methods were largely supplanted with the introduction of yolk sac culture for the preparation of vaccine in the early 1940's.

Of these the Weigl vaccine is impractical for use on any scale. Vaccine prepared from rat lung culture has been used to a considerable extent in Mexico and by the German army during World War II; the method of preparation of such vaccine, involving as it does intratracheal inoculation, is inherently highly

dangerous and also relatively expensive.

The vaccine currently used in the United States is yolk sac vaccine, prepared by ether extraction as noted above, and the final preparation is a suspension of formalin-killed epidemic typhus rickettsiae and soluble antigen. There is some reason to believe that the liberation of soluble antigen increases the immunogenic potency of the material. It is standardized on a toxicity basis, *i.e.*, prevention of the toxic effect of inoculated rickettsiae. It has been used in the United States Army, given as a course of three doses at 7- to 10-day intervals, followed by a single dose every 4 to 6 months. The possibility of allergic reactions following inoculation of the egg material has not proved to be important; approximately six to eight million troops received vaccine with only two fatalities attributable to it.

Experimental evidence strongly suggests that a marked degree of immunity is produced by immunization with this inactivated vaccine. There is no good field test of its efficacy, though only a very few cases of mild typhus and no deaths were recorded in the United States Army during World War II in spite of the exposure of many to the disease. There have been a number of instances of infection of vaccinated and unvaccinated laboratory personnel, and it is clearly apparent that immunization substantially modifies a subsequently acquired infection and the resulting disease is mild.

Attenuated Vaccine. Vaccines prepared with attenuated microorganisms of sharply reduced virulence for man are a necessity in those instances in which the inactivated antigen will not produce an effective immunity, *e.g.*, tuberculosis, yellow fever, etc., or are desirable in those cases in which the induced immunity is of a low order as in plague. Multiplication of the attenuated microorganism provides a larger amount of antigen, undenatured by inactivation processes, and possibly additional antigenic substances formed during growth (Chap. 15), but the possibility of reversion to virulence persists in the use of such vaccines.

In the case of *R. prowazeki,* a spontaneous change to avirulence for guinea pigs and other laboratory animals occurred during continuous egg passage of a strain isolated in Madrid and designated strain E. The possibility of this attenuated strain giving a superior effective immunity has been of considerable interest, and has been studied by Russian workers[52, 79] and by Fox and his associates in this country.[19, 20] In the latter studies human volunteers were used and subjected to extensive field trial in Peru where the disease is endemic. Complement-fixing and toxin-neutralizing antibodies are produced in more than 90 per cent of persons inoculated under field conditions. The immune response was shown to be associated with an effective immunity demonstrable by challenge inoculation with virulent *R. prowazeki* and to persist for more than 6 years after immunization. There has been no indication of a reversion of the attenuated strain to virulence, nor do inoculated persons appear to serve as foci of infection.

THE SPOTTED FEVERS

The rickettsial diseases which make up the spotted fever group are considerably more heterogeneous than those of the typhus fever group. Several of them are but poorly understood as yet. All are transmitted by ticks, although some differ immunologically and clinically from Rocky Mountain spotted fever.

Rocky Mountain Spotted Fever. Clinically Rocky Mountain spotted fever resembles typhus fever; the rash is generally more extensive and the nervous

symptoms may be more pronounced, but in areas where both diseases prevail it is exceedingly difficult to distinguish them on clinical grounds alone.

The disease has been known for many years in the Rocky Mountain region in states such as Idaho, Montana, Wyoming, Oregon, and Washington. In 1930, however, it was found in the eastern part of the United States in the South Atlantic states such as Maryland, Virginia, West Virginia, and North Carolina. Although the disease is not limited to these regions, of the 2190 cases reported from 1933 to 1937, 65.5 per cent were from the Mountain and Pacific states and 27.4 per cent from the South Atlantic group; the two areas combined accounted for 93 per cent of the total cases reported in the country. Approximately 200 to 300 cases are reported each year; 219 cases were reported in 1961.

It is customary to speak of two types of the disease, an eastern type and a western type. Both are, however, immunologically identical and are caused by the same rickettsia, *R. rickettsii,* which, in contrast to the typhus rickettsiae, is found within the nucleus of invaded cells. Both are transmitted by ticks, but by different species. The wood tick, *Dermacentor andersoni,* is the vector of the disease in the western states, and the dog tick, *Dermacentor variabilis,* transmits the disease in the Atlantic region. The rabbit tick, *Haemaphysalis leporis-palustris,* disseminates the infection in the animal population. *Amblyomma americanum, A. cajennense, D. occidentalis, Rhipicephalus sanguineus, D. parumapterus,* and *Ornithodorus parkeri* are able to transmit the disease experimentally and are to be regarded as potential vectors. The infection is congenital, *i.e.,* is transmitted from one generation to the next, in *D. andersoni,* but whether it is congenital in the dog tick is not clear. In the first instance, then, there need be no animal reservoir of infection although it is probable that animal reservoirs of infection occur.[47, 58] In the second, the dog may serve as a temporary reservoir of infection. The body louse may be infected experimentally with these and other rickettsiae of the group,[75] but seems to play little or no part in the human disease.

The case fatality of spotted fever is highly variable. In Idaho it has been about 4 per cent, in western Montana about 20 per cent, and in certain regions of the Bitterroot Valley it is as high as 90 per cent. Over the country as a whole, however, the rate is 18 to 19 per cent. It is generally said that the disease is milder in the Atlantic region than in the Mountain states, but over a period of years there is little difference in case fatality; for the period of 1933 to 1937 the case fatality in the Mountain and Pacific states was 19.4 per cent and for the South Atlantic states 18.1 per cent.

Spotted fever was transmitted to guinea pigs and monkeys by Ricketts in 1907. Guinea pigs show a febrile reaction, the spleen is enlarged, and the necrotic scrotal reaction occurs. *R. rickettsii* is immunologically related to the typhus rickettsiae, but the titer of cross-reaction in the complement-fixation test is not high enough to cause confusion; there is also a small degree of cross-protection between the two.

Other Spotted Fevers. Closely similar tick-borne disease has been observed in Central and South America; in Central America and Brazil it is known as São Paulo typhus, and in Colombia as Tobia fever. The causative rickettsiae are very closely related to, if not identical with, *R. rickettsii.* There seems to be no doubt that these diseases are spotted fever, and there is some tendency to refer to them collectively as American spotted fever.

Immunization. A solid immunity is associated with recovery from spotted fever, and an effective prophylactic immunity may be produced by inoculation with vaccine. The first vaccine was that prepared by Parker and his associates at the United States Public Health Service laboratory in Montana. It consisted of infected tick tissue inactivated with phenol and was used for more than 15 years. Subsequently it was displaced by formalin-inactivated vaccine of rickettsiae grown in yolk sac culture. These

vaccines produce an effective immunity in experimental animals such as guinea pigs, and, while the incidence of the disease is too low to allow adequate field test, there is evidence that the naturally acquired disease in man is either prevented or modified by prophylactic immunization.

RELATED RICKETTSIAL INFECTIONS

There are a number of diseases of rickettsial etiology which are closely related to the American spotted fevers, which are known as tick typhus or tick fever and identified by the locality in which they are observed. The causative rickettsiae have been shown in some cases to be closely related to, but apparently not identical with, *R. rickettsii*, while others which are not this well known show clinical and epidemiological features which suggest a closely similar etiology.

Boutonneuse Fever. This disease, also known as fièvre boutonneuse and Marseilles fever, is prevalent in the Mediterranean area, and is caused by *R. conori*. This rickettsia is immunologically related to, but separable from, *R. rickettsii* by toxin neutralization[8] as well as by other serological methods. Cross-protection is practically complete in convalescent guinea pigs, but immunization with *R. conori* vaccine will not protect against *R. rickettsii* infection, and immunological differences are also demonstrable by the complement-fixation test.

The animal reservoir of infection is the dog, and the disease is transmitted by the tick, *Rhipicephalus sanguineus*, in which the infection is congenital. The disease is milder than American spotted fever, with a case fatality rate of 1 to 2 per cent. It is distinguished clinically by the small, local, indurated lesion with a necrotic center which develops at the site of the tick bite,[34] and is known as the eschar or tâche noire, and by regional adenopathy.

Other tick fevers. The tick-bite fevers of Africa, including Kenya fever or Kenya typhus and South African tick fever, together with Indian tick typhus, appear to be substantially the same disease as boutonneuse fever, although there is no eschar in Kenya fever. In Africa the infection is found in a number of ticks, including *Haemaphysalis leachii, Amblyomma haebreum, Rhipicephalus appendiculatus,* and *R. sanguineus,* and the last is found infected in India also. Kenya fever is transmitted by *R. sanguineus,* but more often by the larvae of *A. haebreum.* While these vectors constitute a reservoir of infection, there is also evidence that a reservoir may be present in wild rodents.

North Queensland Tick Typhus. This disease, occurring in Australia, was first described in 1946. The causative rickettsia is immunologically related to the spotted fever rickettsiae but is sufficiently distinct that it has been designated *R. australis.* The evidence for tick transmission is as yet circumstantial but suggests that *Ixodes holocyclus* is a vector of the disease.

Siberian Tick Typhus. This is a relatively mild rickettsial disease, occurring in Central Asia and extending to the eastern coast of Siberia. The causative rickettsia is closely related to *R. conori,* but differentiable from it,[24] and has been called *R. sibericus.* The disease is believed to be transmitted by several ticks, including *Haemaphysalis concinna, Dermacentor sylvarum,* and *D. nuttallii.*

RICKETTSIALPOX

Rickettsialpox was first observed in New York City in 1946, when 124 cases were reported, and subsequently has been found in urban areas on the eastern seaboard of this country. It has also been reported to occur in urban areas in Russia and has been isolated from wild rodents in Korea.[30] The disease is a mild febrile illness, characterized by a local lesion at the site of infection, and a varicelliform rash. It is rarely if ever fatal.

The causative agent is a rickettsia of the spotted fever group which is immunologically related to *R. rickettsii,* but may be distinguished from it; it has been given the name *R. akari.* The animal reservoir of infection is the wild house mouse, *Mus musculus,* and the disease is transmitted by the mite *Allodermanyssus sanguineus.* The causative agent has been isolated from mice and mites as well as from patients' blood, and the human infection is apparently acquired from the bite of infected mites. *R. akari* is pathogenic for both wild and laboratory mice and for the guinea pig. A fatal disease is produced in the mouse following intraperitoneal inoculation; the infection may be transmitted by spleen or brain, and the rickettsiae are demonstrable on microscopic examination of scrapings from the peritoneal cavity, as from the surface of the spleen. A febrile disease and scrotal reaction are produced in the guinea pig.

R. akari is readily cultivable in the embryonated hen's egg, in the amnionic cavity or the yolk sac. Yolk sac antigen fixes complement in the presence of patient's serum, but also cross-reacts with spotted fever antibody.

TSUTSUGAMUSHI DISEASE (SCRUB TYPHUS, MITE TYPHUS)[18, 41, 65]

A disease of rickettsial etiology prevalent in the Far East is known as tsutsugamushi disease (dangerous bug disease), Japanese flood fever, or Kedani fever in Japan, as mite typhus in Sumatra, and as rural typhus or scrub typhus in Malaya. The last two are not to be confused with shop typhus of Malaya which is murine typhus. The disease is very similar to spotted fever in its distribution and clinical picture but differs in the latter respect in that there is a primary sore and adenitis as in fièvre boutonneuse, and the symptoms include headache, orbital pain, a maculopapular rash, and fever. As in spotted fever, the case fatality rate varies widely, from 0.5 per cent to perhaps as high as 60 per cent in some areas such as Korea. The disease is characterized immunologically by a Weil-Felix reaction in which the O antigen of the OX-K strains of Proteus are agglutinated but the OX-19 strains are not.

The causative microorganism has been variously named *R. tsutsugamushi, R. nipponica, R. orientalis,* and *R. akamushi;* the first, *R. tsutsugamushi,* is the name commonly used. Strains are closely related immunologically but are readily differentiated by complement fixation. There seems, however, to be no clear serological differentiation into types, and the strains also differ in virulence and in degree of adaptation to egg culture. These rickettsiae are apparently quite unrelated to *R. sennetsui,*[5, 21] isolated in Japan from a glandular fever type of disease in man.

The strains commonly used as antigens are the Gilliam and Karp strains, and sometimes the Seerengayee strain; serums from some cases are predominantly of one type or another, while others react to about the same titer with all three. The Gilliam strain is toxic to mice while the others are not, and the toxicity is not neutralized by antiserums to other strains of *R. tsutsugamushi,* or to only a slight degree. *R. tsutsugamushi* may be grown in the yolk sac or chorioallantois of the developing hen's egg, in tissue culture, and in the lungs of rats. Since this rickettsia occurs in the emulsion phase in ether extraction of yolk sac culture, the material may be purified by extraction before saline dilution or by differential centrifugation for the preparation of complement-fixing antigen.

Experimental animals recovered from nonfatal infection show practically complete cross-immunity among strains of *R. tsutsugamushi.* Immunization with

vaccine is a difficult matter, however, for apparently subcutaneous inoculation does not protect, but intraperitoneal vaccine protects against intraperitoneal challenge. Formalinized tissue culture vaccine or rat lung vaccine has been used for successful immunization of experimental animals. Killed vaccines are ineffective in man, but Smadel and his co-workers[37, 61, 62] have produced an effective immunity with living vaccine given together with chloramphenicol to provide protection against infection. At present, however, active prophylactic immunization of man is not practical because of lack of cross-protection between the various strains of the rickettsiae.

Tsutsugamushi occurs over a wide area in the Asiatic Pacific region, including Japan, Korea, Formosa, Indo-China, Malaya, Burma, Assam, New Guinea, and the Philippine Islands. It appears to persist as an infection of rodents and of mites and is transmitted to man by mites. The vertebrate host and insect host and vector differ somewhat from place to place. In Japan the rodent host is the field mouse, *Microtus montebelli,* and the disease is transmitted by the larvae of *Trombicula akamushi.* In Sumatra the vertebrate hosts are the house rat, *Mus concolor,* and the field rat, *Mus diardii,* and in Assam and Burma the wild rat, *Rattus flavipectus yunnanensis,* and the tree shrew, *Tupaia belangeri versurae.* Except in Japan the most important insect vectors appear to be *Trombicula deliensis,* a mite very closely related to *T. akamushi, Trombicula walchi* which is regarded by some as identical with *T. deliensis,* and *Trombicula fletcheri.* The mites occur in low, damp areas of grass, underbrush, and scrub, in Japan along uncultivated banks of rivers, and the infection is thus restricted to certain localities. In the temperate climate of Japan the disease has a seasonal incidence, but in tropical regions there is no strict seasonal distribution of the disease in man.

Q FEVER [40, 55]

Q fever* was described in Australia in 1937 as occurring in slaughterhouse workers, and the causative agent was shown to be a rickettsia. What proved to be a closely similar, if not identical, rickettsia was isolated by Davis in the United States from ticks, and the disease it produced in guinea pigs was called nine-mile fever. When the Australian and American strains proved to be substantially identical, the disease was called American Q fever in this country, but, since the microorganism has been found to be of world-wide occurrence, the geographical designations have been dropped.

This microorganism is set apart from the other rickettsiae in a number of its characteristics. The American strains were found to pass bacteria-proof filters, and the rickettsia was named *R. diaporica* for that reason, but the name *R. burnetii* given the Australian strains had priority. It fails to form a toxin, is more resistant to heat and drying than the other rickettsiae, and is unique within the group, in that arthropod transmission is not an essential link in its dissemination. It is immunologically distinct from the other rickettsiae, but there are minor antigenic differences between strains. In general, it appears to be sufficiently different from the other rickettsiae causing human disease to justify giving it independent generic status, and it is known as *Coxiella burnetii.*

Like the other rickettsiae, it grows profusely in the yolk sac of the embryonated hen's egg and may be cultivated in various kinds of tissue cultures.

* The assumption that Q fever refers to "Queensland fever" is a misconception; in the original reports it refers to "Query fever."

Guinea pigs may be infected, and the infection is either symptomless or accompanied by a febrile reaction, but there is a specific immunological response. There appear to be two kinds of antigens present in *C. burnetii* strains, distinguished by differences in the time required to reach peak titer of complement-fixing antibody in the infected person. Complement-fixing antigen is grown in the yolk sac, and it is important to use a strain which will react with both kinds of antibody; the Henzerling and nine-mile strains have been used extensively for this purpose.

Disease in Man. The disease in man is a febrile respiratory illness, with an incubation period of 14 to 26 days, and the infection is usually acquired by inhalation of infectious material. It characteristically takes the form of atypical pneumonia and is responsible for an appreciable portion of illness diagnosed clinically as primary atypical pneumonia (see also Chaps. 36 and 38), with pneumonic involvement demonstrable by x-ray examination in even mild cases. There is often evidence of liver involvement, headache is a predominant symptom, and there is no rash, but convalescence is apt to be protracted. Complement-fixing antibody is present in convalescent serums, and a hypersensitivity to intradermal vaccine may develop.[32]

The human disease is widespread, occurring in all major geographical areas,[9] as shown both by diagnosed illness and by the prevalence of serum antibody found in surveys. The largest number of cases has been reported from this country and various parts of Europe, especially in the Mediterranean area where the disease is known as epidemic hiberno-vernal bronchopneumonia or Balkan grippe, and occurred in Allied military forces in that area during World War II.

Infection in Lower Animals. The Australian studies showed the existence of a relatively intricate host-parasite relationship. There *C. burnetii* is maintained in wild animals, especially the bandicoot (*Isoodon macrourus*), and is transmitted by the ticks *Haemaphysalis humerosa* and *Ixodes holocyclus*. Cattle may be infected by these ticks, and in turn the cattle ticks *Boophilus microplus* and *H. bispinosa* may become infected. The rickettsiae occur in large numbers in the feces of infected ticks and survive for long periods in the dry state. The contamination of cattle hides with such infected feces, and the inhalation of this dust, is regarded as the probable mechanism of infection of slaughterhouse workers in Australia. The disease also occurs among slaughterhouse workers in this country, but the way in which the infection is contracted is uncertain.

Infection in dairy cattle is very widespread over the United States, with herd infection rates ranging from 1 to 65 per cent.[39] *C. burnetii* is constantly present in the milk from such herds and is shed into the environment in infected placentas at parturition. In spite of the presence of the rickettsia in milk, the ingestion of raw milk does not seem to be related significantly to the occurrence of the human disease, and other methods of transmission, seem to be more important.[29] Pasteurization of milk suffices to destroy the rickettsiae, but the margin of safety is very narrow, and the microorganism is occasionally found in pasteurized milk.[17]

The observations of Lennette and his colleagues have shown that in northern California the infection is maintained in sheep herds by contamination of the environment by milk and infected placentas taken at lambing time, and is reflected in a seasonal increase in human disease. It is probable that infection occurs by inhalation of contaminated dust. Similar observations have been made elsewhere.[42, 43, 63] There is also serological evidence[64] for the occurrence of reservoirs of infection in domestic fowl and wild birds, and their ectoparasites.

TRENCH FEVER

In the course of the First World War a specific infection became known by the name of trench fever or Wolhynian fever. It is said to have caused almost one third of all the illness in some of the armies in northern France and occurred also in Mesopotamia and Saloniki. It appeared again in the Second World War in the German army in Russia.[31] It has a long incubation period (6 to 22 days); the most constant symptom is pain in the legs; the fever is often high and of the relapsing type. Recovery is the rule. The disease can be transmitted to healthy men by the intravenous injection of whole blood taken from patients up to the fifty-first day of the disease.

Natural transmission is chiefly, if not solely, through the body louse. It has been suggested[74] that the disease may be diagnosed by demonstration of rickettsiae in the stomach contents of lice allowed to feed upon the infected individual, and use of such rickettsiae for microscopic agglutination by patients' serum. The bites of infective lice appear to be the main vector in producing trench fever, but the virus is also present in the excreta and may enter through abrasions in the skin caused by scratching. The virus is present in the urine of patients and is said to remain active for a long time in dry louse feces and dried urine.

The studies of Arkwright, Bacot, and Duncan[4] established the fact that in most cases lice or the excreta of lice that had been proved by inoculation to contain the virus also contained large numbers of rickettsiae. Bacot, who became accidentally infected with trench fever, had for over 2 years previously been feeding upon his person a stock of lice known to be free from rickettsiae. Two days after the onset of his attack, he resumed the feeding of these lice, and rickettsiae were observed 8 days afterward in enormous numbers in their excreta. Infection of the lice with rickettsiae continued possibly for as long as 3 months after the disappearance of all symptoms. Nothing, however, is known certainly about the occurrence of rickettsiae in the bodies of patients with trench fever. Further evidence is necessary to establish the causal relation of *R. pediculi* (also called *R. quintana*) to trench fever, although there is a strong presumption that such a connection obtains. The disease was not reproduced in experimental animals.

LABORATORY DIAGNOSIS OF RICKETTSIAL DISEASE[3]

Laboratory diagnosis is an indispensable adjunct to the identification of disease of rickettsial etiology and involves two general kinds of procedures; the one isolation and identification of the causative agent, and the other serological identification of serum antibody. The latter also allows retrospective diagnosis and estimation of prevalence of infection by surveys for antibody.

The isolation of rickettsiae is of limited diagnostic utility because of technical complications and inherent dangers. Guinea pigs and mice are the animals of choice and may be inoculated intraperitoneally with blood taken during the febrile stage of the disease. As described earlier, there may be little overt evidence of infection in the guinea pig with some kinds of rickettsiae, and blind passage, using spleen emulsion as the inoculum, may be required. In addition, blood may be taken and examined for serum antibody, indicating an immune response in symptomless infection.

The antibody response may be assayed nonspecifically by the Weil-Felix reaction, and specifically, using rickett-

sial antigen, by complement-fixation, rickettsial agglutination, toxin neutralization except in the case of *C. burnetii*, and protection of experimental animals against known challenge inoculum. Of these, the last two are not practical for routine application, and by far the most commonly used serological reactions are the Weil-Felix reaction, which almost any laboratory may carry out, and the complement-fixation and/or rickettsial agglutination reactions. The first serves to identify or exclude the broad groups of rickettsiae but is diagnostic for the tsutsugamushi group; the complement-fixation reaction is group-specific but may be made specific or relative species titers may be diagnostic; and rickettsial agglutination is applicable in field studies and is regarded as more species-specific in some instances, *e.g.*, the typhus fevers, than the unmodified complement-fixation reaction.

Weil-Felix Reaction. The agglutination of OX-19, OX-2, and OX-K strains of Proteus allows provisional identification of the typhus and tsutsugamushi or scrub typhus groups of rickettsial disease but does not differentiate among the other rickettsial infections, and irregular results may occur within the so-called indeterminate, or Weil-Felix negative, group of infections.

Normal serums from persons living in endemic localities often show an agglutinin titer of 1:50, and titers as low as this are regarded as negative; 1:100 as suggestive, and 1:200 as diagnostic. The Weil-Felix reaction shows an anamnestic response in a number of febrile diseases, especially typhoid fever. In typhoid fever, however, the titer of the Weil-Felix reaction does not continue to rise after the first week, whereas typhoid bacillus agglutinin continues to rise in titer. In rickettsial disease the agglutinin titer appears relatively early, reaching a titer as high as 1:400 by the end of the first week, and rising to 1:1600 or above by the end of the second week. It drops thereafter, falling to 1:400 or less by the eighth or ninth week after onset, and has no utility in retrospective serodiagnosis. Though classified as an indeterminate type, spotted fever frequently shows a considerable agglutinin titer for Proteus, and differentiation from murine typhus may be difficult when the two diseases occur in the same area.

Specific Serological Reactions. Although in certain instances, notably tsutsugamushi disease, Proteus agglutination suffices; in general complement-fixation, carried out with purified rickettsial antigen, is the serological method of choice, and rickettsial agglutination is equally specific.

In epidemic typhus fever, the complement-fixing antibody titer shows an eightfold rise in titer against the specific antigen between the first and third weeks, and is usually fourfold or greater than the titer observed to *R. typhi* antigen in unimmunized persons. Similarly, in murine typhus the specific complement-fixing antibody titer rises from less than 1:10 in the first week to 1:60 or more by the third week and is consistently higher than titers to *R. prowazeki* antigen in unimmunized individuals. Rickettsial agglutinin titers of 1:200 are regarded as diagnostic in both diseases. It may be extremely difficult to distinguish between the two by serological reactions when the disease occurs in previously immunized persons.[23]

The complement-fixation titer rises about eightfold from the first to the third weeks of the disease in spotted fevers, to a titer of 1:80 or higher. A similar complement-fixing antibody titer rise occurs in rickettsialpox, but a positive Weil-Felix reaction is given with the OX-19 or OX-2 strains of Proteus in spotted fever, but not in rickettsialpox. An approximately similar rise in complement-fixing antibody titer to *C. burnetii* occurs in Q fever, usually without confusing cross-reactions.

REFERENCES

1. Abd-el-Messih, G.: Subclinical infections of epidemic typhus in Egypt. Their origin and magnitude. J. Egyptian Pub. Health Assn., 1960, **35**:35–62.
2. Allen, E. G., Bovarnick, M. R., and Snyder, J. C.: The effect of irradiation with ultraviolet light

on various properties of typhus rickettsiae. J. Bact., 1954, **67:**718–723.
3. American Public Health Association: Diagnostic Procedures for Virus and Rickettsial Disease. American Public Health Association, New York. 2nd ed., 1956.
4. Arkwright, J. A., Bacot, A., and Duncan, F. M.: The association of rickettsia with trench fever. J. Hyg., 1919–20, **18:**76–94.
5. Babudieri, B.: Recherches expérimentales sur *Rickettsia sennetsui,* Misao et Kabayshi, 1956. Arch. Inst. Pasteur de Tunis, 1959, **36:**577–583.
6. Bell, E. J., and Philip, C. B.: The human rickettsioses. Ann. Rev. Microbiol., 1952, **6:**91–118.
7. Bell, E. J., and Pickens, E. G.: A toxic substance associated with the rickettsias of the spotted fever group. J. Immunol., 1953, **70:**461–472.
8. Bell, E. J., and Stoenner, H. G.: Immunologic relationships among the spotted fever group of rickettsias determined by toxin neutralization tests in mice with convalescent animal serums. J. Immunol., 1960, **84:**171–182.
9. Berge, T. O., and Lennette, E. H.: World distribution of Q fever; human, animal and arthropod infection. Amer. J. Hyg., 1953, **57:** 125–143.
10. Bovarnick, M. R.: Phosphorylation accompanying the oxidation of glutamate by the Madrid strain of typhus rickettsiae. J. Biol. Chem., 1956, **220:**353–362.
11. Bovarnick, M. R., and Allen, E. G.: Reversible inactivation of typhus rickettsiae at 0° C. J. Bact., 1957, **73:**56–62.
12. Bovarnick, M. R., and Allen, E. G. Reversible inactivation of the toxicity and hemolytic activity of typhus rickettsiae by starvation. J. Bact., 1957, **74:**637–645.
13. Bozeman, F. M., et al.: Study on the growth of rickettsiae. I. A tissue culture system for quantitative estimations of *Rickettsia tsutsugamushi.* J. Immunol., 1956, **76:**475–488.
14. Burgdorfer, W., and Lackman, D.: Identification of *Rickettsia rickettsii* in the wood tick, *Dermacentor andersoni,* by means of fluorescent antibody. J. Infect. Dis., 1960, **107:**241–244.
15. Cohn, Z. A.: Relation of cell metabolism to infection with rickettsiae and bacterial agents. Bact. Rev., 1960, **24:**96–105.
16. Cox, H. R.: Viral and rickettsial toxins. Ann. Rev. Microbiol., 1953, **7:**197–218.
17. Enright, J. B., Sadler, W. W., and Thomas, R. C.: Thermal inactivation of *Coxiella burnetii* and its relation to pasteurization of milk. Public Health Service Publication No. 517. Public Health Monograph No. 47. U. S. Department of Health, Education and Welfare, 1957.
18. Farner, S. D., and Katsampes, C. P.: Tsutsugamushi disease. U. S. Naval Med. Bull., 1944, **43:**800–836.
19. Fox, J. P.: A review of experience with an avirulent strain of *R. prowazeki* (strain E) as a living agent for immunizing man against epidemic typhus. Amer. J. Pub. Health, 1955, **45:** 1036–1048.
20. Fox, J. P., Jordan, M. E., and Gelfand, H. M.: Immunization of man against epidemic typhus by infection with avirulent *Rickettsia prowazekii* strain E. IV. Persistence of immunity. J. Immunol., 1957, **79:**348–354.
21. Giroud, P., Capponi, M., and Dumas, N.: Comparison entre *Rickettsia sennetsui* et *Rickettsia tsutsugamushi:* morphologie, pouvoir pathogène, pouvoir antigénique. Bull. Soc. Path. Exot., 1960, **53:**960–965.
22. Goldwasser, R. A., and Shepard, C. C.: Fluorescent antibody methods in the differentiation of murine and epidemic typhus sera; specificity changes resulting from previous immunization. J. Immunol., 1959, **82:**373–380.
23. Goldwasser, R. A., Shepard, C. C., Jordan, M. E., and Fox, J. P.: Specificity of antibody response in typhus fever. Its alteration during murine typhus infection as a result of previous exposure to epidemic typhus antigen. J. Immunol., 1959, **83:**491–495.
24. Golinevitch, H.: A propos de la différenciation de quelques rickettsies de groupe de la fièvre pourprée tiques. Arch. Inst. Pasteur de Tunis, 1960, **37:**13–22.
25. Guardiola, A. L., and Paretsky, D.: Metabolic reactivation of rickettsiae by diphosphopyridine nucleotide. Science, 1958, **128:**141–142.
26. Hayes, J. E., et al.: Metabolic studies of rickettsiae. Biochim. Biophys. Acta, 1957, **26:**570–576.
27. Hopps, H. E., et al.: Metabolic studies of rickettsiae. III. Studies of transamination, oxidative phosphorylation and glutamate 2-C^{14} incorporation by purified suspensions of *Rickettsia mooseri.* J. Bact., 1956, **71:**708–716.
28. Hoyer, B. H., et al.: Mammalian viruses and rickettsiae. Science, 1958, **127:**859–862.
29. Huebner, R. J., and Bell, J. A.: Q fever studies in southern California. Summary of current results and a discussion of possible control measures. J. Amer. Med. Assn., 1951, **145:**301–305.
30. Jackson, E. B., Danauskas, J. X., Coale, M. C., and Smadel, J. E.: Recovery of *Rickettsia akari* from the Korean vole *Microtus fortis pelliceus.* Amer. J. Hyg., 1957, **66:**301–308.
31. Jacobi, J.: Neue Beobachtungen über Fünftagefieber mit besonderer Berücksichtigung der Differentialdiagnose und Therapie. Münch. med. Woch., 1942, **89:**615–618.
32. Lackman, D. B., Bell, E. J., Bell, J. F., and Pickens, E. G.: Intradermal sensitivity testing in man with a purified vaccine for Q fever. Amer. J. Pub. Health, 1962, **52:**87–93.
33. Lawy, H. S., Beattie, C. P., and Bensted, H. J.: Brill-Zinsser disease; the possibility of its occurrence in Britain. J. Hyg., 1958, **56:**355–363.
34. Le Gac, P., et al.: L'escarre d'inoculation du typhus des broussailes indochinois. Bull. Soc. Path. Exot., 1959, **52:**263–264.
35. Levina, E. N., and Balaeva, N. M.: Detection of rickettsias by means of fluorescent antibodies. J. Microbiol. Epidemiol. Immunobiol., 1960, **31:**2013–2016. (Translated from the Russian.)
36. Lewthwaite, R.: The typhus group of fevers. Brit. Med. J., 1952, **ii:**826–828.
37. Ley, H. L., Jr., et al.: Immunization against scrub typhus. IV. Living Karp vaccine and chemoprophylaxis in volunteers. Amer. J. Hyg., 1952, **56:**303–312.
38. Love, G. J., and Smith, W. W.: Murine typhus investigations in southwestern Georgia. Pub. Health Repts., 1960, **75:**429–440.
39. Luoto, L.: Report on the nationwide occurrence of Q fever infections in cattle. Pub. Health Repts., 1960, **75:**135–140.
40. Luoto, L., and Pickens, E. G.: A resume of recent research seeking to define the Q fever problem. Amer. J. Hyg., 1961, **74:**43–49.
41. Mackie, T. T., et al.: Observations on tsutsuga-

mushi disease (scrub typhus) in Assam and Burma; preliminary report. Amer. J. Hyg., 1946, **43:**195–218.
42. Marmion, B. P., and Harvey, M. S.: The varying epidemiology of Q fever in the southeast region of Great Britain. I. In an urban area. J. Hyg., 1956, **54:**533–546.
43. Marmion, B. P., and Stoker, M. G. P.: The epidemiology of Q fever in Great Britain. An analysis of the findings and some conclusions. Brit. Med. J., 1958, **ii:**809–816.
44. Myers, W. F., and Paretsky, D.: Synthesis of serine by *Coxiella burnetii.* J. Bact., 1961, **82:** 761–763.
45. Paretsky, D., Consigli, R. A., and Downs, C. M.: Studies on the physiologie of rickettsiae. III. Glucose phosphorylation and hexokinase activity in *Coxiella burnetii.* J. Bact., 1962, **83:** 538–543.
46. Pastukhova, G. M.: The epidemiological characteristics of primary and recurrent typhus. J. Microbiol. Epidemiol. Immunobiol., 1957, **28:** 1307–1310. (Translated from the Russian.)
47. Philip, C. B.: Some epidemiological considerations in Rocky Mountain spotted fever. Pub. Health Repts., 1959, **74:**595–600.
48. Pinkerton, H.: The pathogenic rickettsiae with particular reference to their nature, biological properties and classification. Bact. Rev., 1942, **6:**37–78.
49. Price, W. H.: The epidemiology of Rocky Mountain spotted fever. I. The characterization of strain virulence of *Rickettsia rickettsii.* Amer. J. Hyg., 1953, **58:**248–268.
50. Price, H. H.: A quantitative analysis of the factors involved in the variations in virulence of rickettsae. Science, 1953, **118:**49–52.
51. Price, W. H., et al.: Ecologic studies on the interepidemic survival of louse-borne epidemic typhus fever. Amer. J. Hyg., 1958, **67:** 154–178.
52. Pshenichov, V. A., Levashov, A. A., and Nikolenko, B. I.: Biological characteristics of the vaccine strain E of *Rickettsia prowazeki.* Observations on the immunization of human subjects with live typhus vaccine. Problems Virol., 1959, **4:**64–70. (Translated from the Russian.)
53. Public Health Service: Studies of typhus fever. National Institute of Health Bull. No. 183. U. S. Public Health Service, Federal Security Agency, 1945.
54. Quinley, G. E., and Schubert, J. H.: Epidemiologic and serologic appraisal of murine typhus in the United States, 1948–1951. Amer. J. Pub. Health, 1953, **43:**160–164.
55. Reusse, U.: Die Bedeutung des Q-Fiebers als Zoonose. Ztschr. f. Tropenmed. u. Parasit., 1960, **11:**223–262.
56. Schaechter, M., et al.: Morphological, chemical and serological studies of cell walls of *Rickettsia mooseri.* J. Bact., 1957, **74:**822–829.
57. Schaechter, M., Bozeman, F. M., and Smadel, J. E.: Study on the growth of rickettsiae. II. Morphologic observations of living rickettsiae in tissue culture cells. Virology, 1957, **3:**160–172.
58. Shirae, A., et al.: Ecology of Rocky Mountain spotted fever. I. *Rickettsia rickettsii* recovered from a cottontail rabbit in Virginia. Proc. Soc. Exptl. Biol. Med., 1961, **107:**211–214.
59. Smadel, J. E.: Chloramphenicol (Chloromycetin) and tropical medicine. Trans. Roy. Soc. Trop. Med. Hyg., 1950, **43:**555–574.
60. Smadel, J. E.: Status of the rickettsioses in the United States. Ann. Intern. Med., 1959, **51:** 421–435.
61. Smadel, J. E., et al.: Immunization against scrub typhus. I. Combined living vaccine and chemoprophylaxis in volunteers. Amer. J. Hyg., 1951, **53:**317–325.
62. Smadel, J. E., et al.: Immunization against scrub typhus. Duration of immunity in volunteers following combined living vaccine and chemoprophylaxis. Amer. J. Trop. Med. Hyg., 1952. **1:**87–99.
63. Stoker, M. G. P., and Marmion, B. P.: The spread of Q fever from animals to man. Bull. W. H. O., 1955, **13:**781–806.
64. Syruovchek, L., and Raska, K.: Q fever in domestic and wild birds. Bull. W. H. O., 1956, **15:**329–337.
65. Tarasevitch, I. V.: Tsutsugamushi fever (a review). J. Microbiol. Epidemiol. Immunobiol., 1960, **31:**1581–1590. (Translated from the Russian.)
66. Tonge, J. I.: Brill's disease (recrudescent epidemic typhus) in Australia. Med. J. Australia, 1959, **2:**919–921.
67. Topping, N. H., and Shepard, C. C.: The rickettsiae. Ann. Rev. Microbiol., 1947, **1:**333–350.
68. Weiss, E.: Some aspects of variation of rickettsial virulence. Ann. N. Y. Acad. Sci., 1960, **88:** 1287–1297.
69. Weiss, E., and Dressler, H. R.: Growth of *Rickettsia prowazeki* in irradiated monolayer cultures of chick embryo entodermal cells. J. Bact., 1958, **75:**544–552.
70. Weiss, E., and Dressler, H. R.: Selection of an erythromycin-resistant strain of *Rickettsia prowazekii.* Amer. J. Hyg., 1960. **71:**292–298.
71. Weiss, E., Dressler, H. R., and Suitor, E. C., Jr.: Selection of a mutant strain of *Rickettsia prowazekii* resistant to *p*-aminobenzoic acid. J. Bact., 1957, **73:**421–430.
72. Weiss, E., Dressler, H. R., and Suitor, E. C., Jr.: Further studies of drug-resistant strains of *Rickettsia prowazekii.* J. Bact., 1959, **77:**91–100.
73. Weiss, E., and Pietryk, H. C.: Growth of *Coxiella burnetii* in monolayer cultures of chick embryo entodermal cells. J. Bact., 1956, **72:** 235–241.
74. Werner, H.: Zur Diagnose des Fünftagefiebers. Deut. med. Woch., 1942, **68:**934–935.
75. Weyer, F.: Weitere Beobachtungen und Versuche über das Verhalten von Rickettsien in Kleiderläusen. Ztschr. f. Tropenmed. u. Parasit., 1961, **12:**97–117.
76. Wisseman, C. L., Jr., et al.: Studies on rickettsial toxins. V. Dissimilarity between the action of rickettsial mouse lethal toxin and bacterial endotoxin in mice. J. Immunol., 1961, **86:**613–617.
77. Wissig, S. L., Caro, L. G., Jackson, E. B., and Smadel, J. E.: Electron microscopic observations on intracellular rickettsiae. Amer. J. Path., 1956, **32:**1117–1133.
78. Yamamoto, T., Kawamura, A., Jr., Hara, H., and Aikawa, K.: Partial purification of rickettsiae with a cation exchange resin. I. Method of purification. Japanese J. Exptl. Med., 1958, **28:**329–336.
79. Zdrokovskii, P. F.: The problem of live typhus vaccine. Problems Virol., 1958, **3:**141–145. (Translated from the Russian.)

Chapter 36

THE PSITTACOSIS–LYMPHOGRANULOMA VENEREUM GROUP OF VIRUSES

THE VIRAL agents making up the psittacosis-lymphogranuloma venereum group[3, 63, 64] resemble one another closely, and it is believed that they may have differentiated from a common ancesteral form by association with different host species. The group includes the psittacosis and ornithosis viruses, mouse pneumonitis virus, feline pneumonitis virus, human pneumonitis viruses such as the Louisiana and San Francisco strains found in primary atypical pneumonia, the virus of opossum meningopneumonitis, and those causing bovine encephalitis and ewe abortion. The virus of trachoma and that of inclusion conjunctivitis are somewhat set apart from the group, although related to it, and are often considered with it.

The viruses of this group resemble the rickettsiae in a number of respects, *i.e.*, they stain similarly and are sufficiently large that they fall just within the limits of optical resolution; there is some evidence that multiplication may include a process resembling binary fission; the diseases they produce are unique among those of viral etiology in that they may be treated effectively with certain antibiotics, and some strains are susceptible to the activity of sulfonamides. Similarities such as these have prompted some workers to group these viruses with the rickettsiae, but they are more appropriately regarded as intermediate forms which constitute a link between the rickettsiae on the one hand, and the viruses proper on the other.

These viruses occur in a variety of vertebrate hosts. Naturally occurring infections with the viruses of lymphogranuloma venereum, trachoma, and inclusion conjunctivitis appear to be confined to man. Psittacosis virus occurs in psittacine birds, and the closely related, if not substantially identical, ornithosis viruses occur in wild birds such as pigeons and gulls, and in domestic fowl, especially the turkey; human infection is most commonly acquired by contact with such reservoirs of infection. Whether the pneumonitis viruses of human origin, having minor but distinctive immunological character, occur primarily in man is uncertain. Pneumonitis virus infections in lower animals are relatively common; the infections are often latent, and it has been suggested that intensive study of apparently normal mice, birds, etc., may show the frequent presence of viruses of this kind.

Morphology and Staining. The mature virus particle, or elementary body, is spherical or coccoidal in shape and relatively large, 200 to 300 mμ in diameter. The particle appears to consist

of a central mass of electron-dense material surrounded by less dense material and enclosed within a limiting membrane. The structure is apparently not markedly rigid and tends to collapse on drying to give the appearance of a particle as much as 500 mμ in diameter. In shadow-cast preparations the more rigid central mass is clearly apparent within the flattened particle. The membrane has been isolated and purified by lysis with desoxycholate and tryptic digestion, and found to resemble closely the cell walls of the gram-negative bacteria and rickettsiae in composition and properties.[37]

Chemical analysis of purified elementary bodies of some of these viruses has shown that, like certain other large viruses such as the pox viruses, they contain protein, lipid, carbohydrate, and both pentose and deoxypentose nucleic acids in proportions similar to those found in bacteria and rickettsiae.[47]

Developmental cycle.[32, 40] The virus occurs in infected tissue as intracytoplasmic inclusion bodies or vesicles, and the morphology of these and the corpuscular elements making them up is variable as a consequence of the sequential occurrence of morphological types during the growth cycle as described elsewhere (Chap. 7). In brief, following penetration of the host cell, the elementary bodies increase in size, to as much as 800 mμ in diameter, to become initial bodies which increase in numbers to form clusters or plaques in a ground substance or matrix. The corpuscular elements increase in number and decrease in size, giving rise to a vesicle containing large numbers of elementary bodies. In living preparations the virus particles are at first immobile, but as the vesicle matures the matrix apparently breaks down and the elementary bodies show brownian movement. Subsequently the vesicle disintegrates, liberating elementary bodies which may then initiate a new infection. This growth cycle is completed in 24 to 48 hours, the time required depending on the virus and the host tissue.

Staining. The elementary bodies resemble rickettsiae in that they stain more readily with aniline dyes than do the other viruses, staining red with the Macchiavello stain and dark purple with the Castaneda stain. The relatively immature inclusion body, containing dense ground substance, is colored blue. As it matures the matrix becomes less dense, the larger virus particles are stained blue, and the smaller elementary bodies red by Macchiavello stain against the pale blue background of the matrix.

Toxin.[17] All of the strains of lymphogranuloma venereum, psittacosis and or-

Figure 259. Shadow-cast electron micrograph of feline pneumonitis virus. Note the collapse of the spherical particle in the dried preparation showing the dense central mass and the outer limiting membrane. × 30,000. (Moulder and Weiss.)

Figure 260. A mature intracellular vesicle of psittacosis virus in chick endodermal cell culture. May-Gruenwald-Giemsa stain; × 432. (Weiss.)

nithosis, and pneumonitis viruses examined have been found to be toxic. The activity is labile and destroyed in a short time at 37° C. or by low concentrations of formaldehyde. It is closely associated with the elementary body and would appear to be an endotoxin rather than an exotoxin. The toxicity is demonstrable by inoculation of mice with heavily infected tissue, commonly yolk sac of the infected embryonated hen's egg, in dilutions of 1:40 or less. Following intravenous inoculation with 0.5 ml. volumes death occurs usually within 24 hours. Because the toxicity has not been separated from living infective virus, two-phase death curves may be observed, *i.e.*, deaths attributable to toxicity occurring within 24 to 30 hours and those from infection beginning at about 48 hours. The common feature at autopsy is liver damage and often necrosis, but damage to the lungs and kidneys may occur also. The toxicity is specifically neutralized by antiserum (see below) but not by chemotherapeutic drugs.

Virus strains may vary considerably in toxicity. In a group of thirty-nine strains, twenty-two of mammalian and seventeen of avian origin, seventeen of the mammalian strains killed in a dilution of 1:10 or greater, including two human pneumonitis strains that killed in a dilution of 1:80, while five of the avian strains failed to kill within 24 hours in dilutions of 1:10 or greater.[41] There is reason to believe that such toxicity may contribute significantly, as in the production of hemorrhage and edema in the lungs, to the pathogenesis of the diseases caused by these viruses.

Antigenicity. Complement-fixing, protective or neutralizing, and antitoxic antibodies to these viruses are demonstrable. Two kinds of antigens, one heat-stable and one heat-labile, are concerned in complement fixation in the presence of convalescent or immune serums, and this reaction is useful for serodiagnostic purposes. Unfortunately, as usually carried out, it is nonspecific, not only with respect to viruses of the psittacosis-lymphogranuloma venereum group proper, but cross-reactions with these viruses are also given by the serums of persons infected with trachoma or inclusion conjunctivitis viruses. With separation of the cell wall, it has been found that the group-specific complement-fixing antigens are intracellular, *i.e.*, present in the lysate, while type-specific antigenicity occurs in the cell wall.[38, 54]

The neutralization or protection test,[16] carried out by incubation of virus with

antiserum prior to inoculation or previous passive immunization of the mouse respectively, was developed by Hilleman and by St. John and Gordon, and the toxin neutralization test by Manire and Meyer.[42] Only small amounts of protective and neutralizing antibody are found in convalescent serums so that such tests have no diagnostic utility. Hyperimmune serums from most laboratory animals are similarly deficient, but such antibody may be prepared to high titer in the rooster and used for differential purposes.[34]

Both of these kinds of tests show a considerable degree of strain specificity. In a general way, infection experiments have suggested that the human pneumonitis strains are relatively specific, the strains of psittacine origin less so, and the meningopneumonitis (human) and pigeon strains have the broadest antigenic structures. More specifically, lymphogranuloma venereum, mouse pneumonitis, and feline pneumonitis viruses have been found to be highly specific in the neutralization test, and, on the basis of antitoxic specificity, Manire and Meyer divided twenty-seven strains into six groups, *viz.*, (1) Louisiana (human) pneumonitis virus; (2) San Francisco (human) pneumonitis virus; (3) feline pneumonitis virus; (4) meningopneumonitis (human) and certain ornithosis viruses; (5) ornithosis virus strains isolated from pigeons; and (6) a group of virus strains of pigeon and human origin.

A hemagglutinin for mouse erythrocytes is formed by viruses of this group, allowing assay of hemagglutin-inhibiting activity.[22] It is not consistently present in infectious allantoic fluid, and is usually of low titer, 1:32 at the most, when present. It seems not to be eluted from mouse red cells, and the antigenicity appears to be nucleoprotein in nature and group-specific.[23]

A passive hemagglutination test, using soluble antigen absorbed on tannic acid–treated sheep red cells,[7] does not differentiate among the ornithosis and pneumonitis viruses, *i.e.*, is group-specific, but the sensitizing antigen is not identical with the complement-fixing antigen.

It is apparent that both common and specific antigens are distributed among these viruses and that different antigens are concerned in these tests. At the same time, neither the details of an antigenic structure nor the implications of host species on the immunological character of these viruses have been precisely defined.

Experimental Infections. Differences in the pathogenicity of these viruses are evident in both the host range of naturally occurring infections and in the relative susceptibility of various experimental animals to infection. With the exception of the viruses of trachoma and

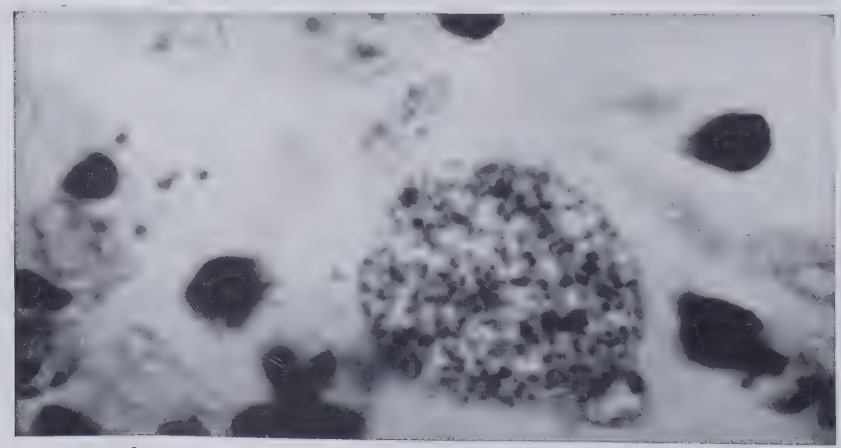


Figure 261. A vesicle containing elementary bodies from the lung of a mouse infected with mouse pneumonitis. Noble's stain; × 2100.

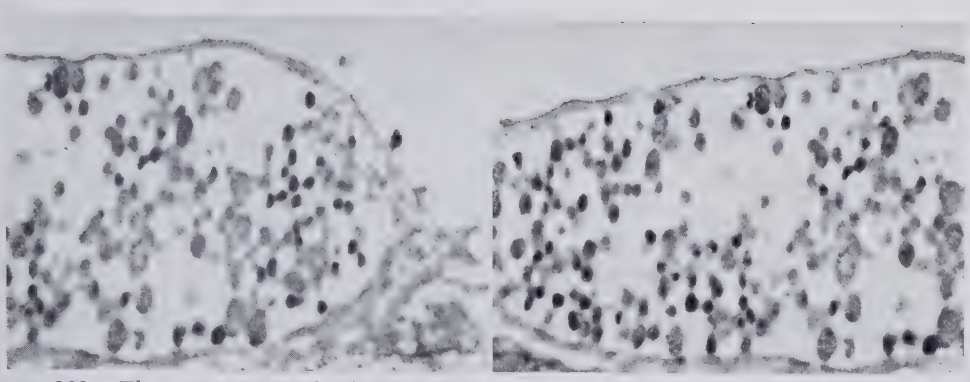


Figure 262. Electron micrograph of mature feline pneumonitis elementary bodies showing the two types of virus particles, the one smaller and dense, and the other larger and granular in structure. × 15,000. (Litwin.)

inclusion conjunctivitis, the mouse may be infected with all of the viruses of this group, and they may be grown in the embryonated hen's egg and various kinds of tissue culture.

Mice may be infected by the intranasal, intraperitoneal, and intracerebral routes to produce fatal infections with most strains of these viruses. Intranasal inoculation results in the development of a pneumonic process characterized by consolidation, and elementary bodies are found in large numbers in impression smears of the affected tissue. At autopsy animals infected by the intraperitoneal route show enlargement of liver and spleen and the presence of fibrous exudate in the abdominal cavity containing large numbers of elementary bodies. A meningoencephalitis with an exudate containing polymorphonuclear and mononuclear cells is produced by intracerebral inoculation. Mice may also be infected by subcutaneous inoculation to give a generalized infection which is less often fatal and usually protracted.

These viruses may be grown in the embryonated hen's egg in the yolk sac where they multiply to high titer, and some grow well in the allantoic cavity or upon the chorioallantoic membrane. Multiplication of feline pneumonitis virus in the yolk sac is associated with a high metabolic rate and an increased rate of synthesis of nucleic acid. According to Moulder and his colleagues[46] the energy for viral replication is supplied by high energy phosphate bonds produced by the aerobic oxidation of endogenous substrates. Studies on the growth of psittacosis virus in chick embryo tissue culture[2, 35, 44, 45] have shown that viral replication is dependent upon the presence of folic and/or folinic acid together with certain purines and amino acids. Although cytochrome C reductase activity[1] and significant amounts of folic acid[14] have been found in purified preparations of meningopneumonitis virus and in feline pneumonitis, mouse pneumonitis, and psittacosis viruses, there is as yet no definitive evidence that these viruses have a capacity for even the limited metabolic reactions demonstrable in rickettsiae.

Chemotherapy.[36] As indicated earlier, the viral agents of this group are unique among the viruses in their susceptibility to certain of the antibacterial chemotherapeutic substances. In general, tetracyclines effectively suppress the growth of these viruses, penicillin has an appreciable but lesser effect, and chloramphenicol and streptomycin are least effective. There is some variation among strains, and the San Francisco and Louisiana viruses and single strains of ornithosis virus of pigeon origin and of meningopneumonitis and feline pneumonitis viruses have been found to be unaffected by sulfadiazine.[27]

The activity of these substances on these viruses is similar to their activity on sensitive bacteria, *i.e.*, they are primarily virustatic. In the case of sulfonamides the activity is antagonized by

p-aminobenzoic acid. Inhibition by penicillin and the tetracyclines is reflected morphologically as an abnormal growth; in the first instance irregular, vacuolated plaques are formed within the infected cell, and in the second the multiplication of the initial bodies is inhibited.[24]

Treatment of infections with these viruses not infrequently results in an immune carrier state. The treated individual continues to harbor, or shed, virus, sometimes for long periods; for example, in one human case of psittacosis for as long as 8 years.

The virustatic effect of chemotherapeutic substances on the viruses of this group has been taken by some to imply the existence of as yet undetected independent metabolic activity of the virus. While this inference may eventually be substantiated by direct, and therefore more convincing, evidence, it is also possible that the effect is, at least in part, one upon the physiological mechanisms of the host cell concerned with viral replication.

Drug resistance. It is of particular interest that a number of these viruses may be made resistant to chemotherapeutic agents by passage in the embryonated egg or mouse in their presence.[25, 48] Such changes are presumably of the same nature as those observed with other microorganisms, and drug resistance has been used as a marker in genetic studies on these viruses.[26, 49]

PSITTACOSIS AND ORNITHOSIS[43]

Psittacosis, or parrot fever, was observed in Switzerland in 1880. For many years it was regarded as an unimportant disease, possibly caused by Nocard's bacillus which is now designated *Salmonella typhimurium*. In 1929 and 1930 it appeared in many parts of the world as a disease acquired for the most part from South American parrots. The elementary bodies were described in 1930 independently by Levinthal in Germany, Coles in England, and Lillie in the United States, and for this reason are sometimes known as LCL bodies. Their viral nature and etiological relation to the disease was established in the early 1930's largely through the work of Bedson and his colleagues.

During this decade it also became apparent that exotic imported psittacine birds are not the only source of infection, and, in fact, that a domestic reservoir of infection occurs in gulls and other sea birds, pigeons, ducks, chickens, etc. The 1938 epidemic of pneumonitis in the Faroe Islands, for example, was associated with widespread infection in fulmar petrels. In the 1940's a variety of similar viruses were isolated from other lower animals and from human cases of pneumonitis.

The disease in man was originally called psittacosis because it appeared to be acquired largely from psittacine birds, and the term ornithosis was introduced when the broader base of the reservoir of infection became apparent. The more inclusive term ornithosis, while not yet universally used, has gained increasing acceptance, especially for disease in and acquired from other than psittacine birds.

The Disease in Man. The disease produced in man by these viruses is essentially the same regardless of the source of infection. The incubation period is 1 to 2 weeks, and the onset may be sudden or insidious. The symptoms include chills and fever, photophobia, headache, anorexia, sore throat, nausea, and vomiting. A dry cough develops which persists and may become more severe, cyanosis and low blood pressure are frequent, and disorientation, apathy, insomnia, and occasionally delirium indicate involvement of the central nervous system. Leucocytosis does not occur until late in the disease or

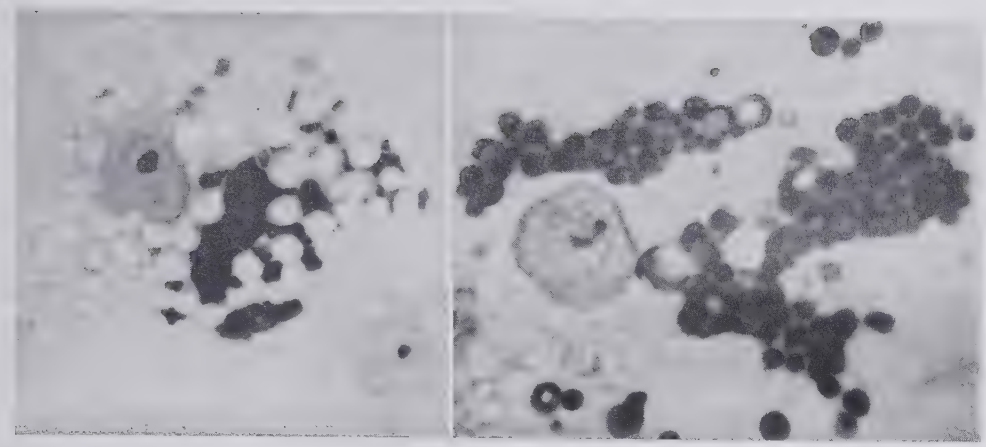


Figure 263. The effect of penicillin on the morphology of feline pneumonitis virus. *Left*, untreated culture; *right*, penicillin-treated. The abnormal morphology in the presence of the drug is apparent. × 600. (Weiss.)

early convalescence, and the extent of the lung involvement is usually not apparent except by x-ray examination, which shows patchy areas of consolidation in one or both lungs. The virus may be found in the blood during the first week of the disease, and in the scanty sputum after the lungs are involved.

At autopsy the areas of consolidation are sharply demarcated from normal tissue, and alveoli containing air, serum, or serofibrous exudate, in which the cellular response is predominantly mononuclear, are dispersed in the areas of consolidation. The exudate cells and those of the hilar lymph nodes contain intracytoplasmic elementary bodies. Other necropsy findings include congestion and focal necrosis in the liver, possible enlargement of the spleen, cloudy swelling in the cardiac muscle, degenerative changes in the kidney parenchyma, and often congestion and edema of the brain and cord. Such findings are clearly indicative of the generalized nature of the infection. The case fatality rate was about 20 per cent in 1929 and 1930, dropped to about 10 per cent with the recognition of mild cases by serodiagnostic methods, and may be further reduced by therapy with tetracyclines.

The Disease in Lower Animals. Naturally acquired infection is not uncommon among certain lower animals and may occur, as a latent infection, even more frequently than has been supposed.

Birds. The natural hosts of these viruses among birds include parrots, parakeets, budgereegahs, etc., totaling not less than thirty-one species of the parrot family. Other birds such as canaries, finches, and sparrows contract the disease when exposed to infected psittacine birds, and naturally occurring latent infection among pigeons appears to be relatively common, and viruses of the same group are found in doves, petrels, gulls, and egrets. The domestic chicken is naturally infected, though not commonly, and spontaneous infection, possibly derived from wild birds such as gulls, and at times assuming epidemic form, occurs in turkeys. There are some differences in virulence and/or susceptibility, depending upon the virus and the host species. For example, viruses of psittacine origin rarely produce fatal meningoencephalitis in pigeons, but viruses from pigeons and chickens usually produce fatal encephalitis on intracerebral inoculation.

In the parrot the disease is characterized by shivering, weakness, apathy, and diarrhea, and respiratory disturbance may be evident. In these birds the disease is an infection of the liver and spleen with occasional involvement of the lungs, and virus is present in the blood, affected tissues, nasal secretions,

and feces. At autopsy of birds dying in the acute stage of the disease the spleen is enlarged and may show areas of focal necrosis, and the liver shows similar changes together with local hemorrhage and infarction, and a semipurulent exudate is found in the pericardial sac and on the air sac and inner lining of the sternum.

Naturally occurring infection in birds is frequently nonfatal, and there is reason to believe that there is a reservoir of latent infection of considerable proportions. Infected birds excrete the virus in the droppings, and dried fecal material is an important source of airborne infection.

Mammals. A number of mammals are infected with closely similar viruses in a latent form, and such infections may be activated to give a clinically apparent, and possibly fatal, disease. This is a point of considerable practical importance in the isolation and study of viruses of this group. The meningopneumonitis virus, for example, which has been used extensively for experimental purposes, was originally isolated from ferrets inoculated intranasally with material from human pneumonitis; it is not clear whether the virus was present in the human disease and so isolated or whether it represented a latent infection in the ferret activated by the inoculation. Similarly, a number of these viruses have been found to occur as latent infections in mice.

Still other viruses of this group have been isolated from respiratory disease in man going under the names of virus pneumonia, pneumonitis, and primary atypical pneumonia. All human diseases of this kind are, of course, not caused by viruses of this group; the Q fever rickettsiae, for example, produce a similar human disease (Chap. 35), and the relative importance of these and other viral agents in acute respiratory infection in man is not precisely known.

Other mammals are naturally infected with viruses of this group, including the virus pneumonia (not influenza) of swine, meningopneumonitis of opossums, bovine encephalitis, ewe abortion, and cat distemper. The last, feline pneumonitis virus, has been widely used for experimental purposes. Of the usual experimental animals, the mouse is susceptible to infection with the viruses of this group by various routes of inoculation, the guinea pig gives little more than a febrile reaction to viruses of psittacine origin, but certain human strains and a strain from egrets are highly virulent for this animal. Some psittacine strains produce a fatal meningoencephalitis in the rabbit on intracerebral inoculation; rhesus monkeys may be infected by the intranasal or intracerebral routes, but rats are relatively resistant to intraperitoneal inoculation. In general, irregular results are obtained with the more resistant animals.

Immunity. As indicated above, the immune response results in the appearance of complement-fixing, protective,

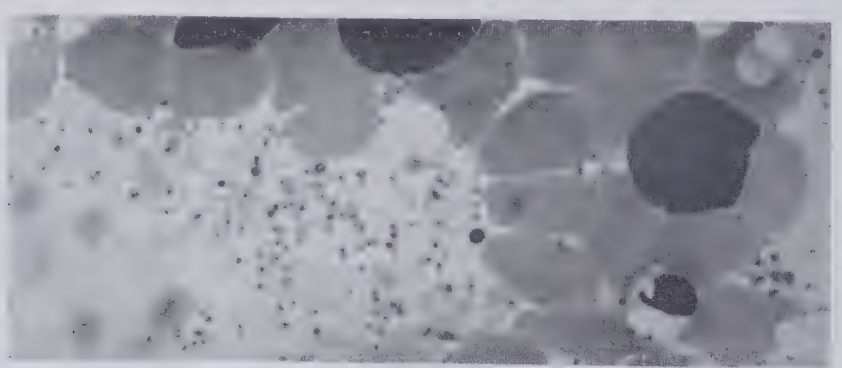


Figure 264. Elementary bodies of psittacosis. Impression preparation from infected mouse spleen; reduced from × 2575. (Bedson.)

and antitoxic antibody activity, varying with the host species. Complement-fixing antibody appears in 10 days to 2 weeks and hypersensitivity a week or two later. Effective immunity appears to be in part an infection immunity and dependent upon the continued presence of the virus. Some attempts have been made to produce a prophylactic immunity in man by the use of vaccines, but with as yet somewhat uncertain results.

Serodiagnosis. Serodiagnosis is dependent in practice on the complement-fixation reaction, carried out with paired serums to demonstrate a rise in titer. The rise occurs in 10 days to 2 weeks but may be delayed until 2 to 5 weeks by chemotherapy; a titer of 1:16, coupled with typical clinical findings, is often considered as positive.

The complement-fixation reaction is dependent upon one or the other of the complement-fixing antigens noted above. The heat-labile antigen is presumably protein in nature since it is inactivated by proteolytic digestion and tends to be strain-specific, and the heat-stable antigen, which is group-specific, is considered to be polysaccharide because it is sensitive to periodate. The specificity of the test may be increased by preliminary absorption of the serum with steamed antigen and using living virus in the complement-fixation test, but the use of living virulent antigen is impractical for routine purposes. Further differentiation between the two may be made on the basis of behavior of complement-fixing antibody titer; serums of persons infected with lymphogranuloma venereum virus react to high titer with psittacosis virus antigen, but the titer declines during convalescence while that of psittacosis patients persists for many weeks or even months. Serological differentiation from certain primary atypical pneumonias is often possible because persons infected with viruses of this group do not usually show the presence of cold agglutinins.

The application of the complement-fixation reaction to the diagnosis of avian infection is complicated in the case of chickens and ducks because immune serums from these birds do not fix complement in the presence of homologous antigen. A complement-fixation inhibition, or indirect complement-fixation, test, using heated antigen, has been devised[39] for use with such serums. In this reaction the fixation of complement by pigeon antibody in the presence of homologous antigen is specifically inhibited in the presence of antibody-containing serums of infected chickens or ducks.

Hypersensitivity. The hypersensitivity developed during infection has been useful for diagnostic purposes in surveys of flocks of domestic birds, especially turkeys. The antigen concerned is separable from complement-fixing antigen by high speed centrifugation, remaining in the supernatant. It may be extracted from infected yolk sac material with sodium lauryl sulfate, is precipitated by dilute acid, purified by butanol fractionation, differs from complement-fixing antigen in that it is soluble in 5 per cent phenol, and appears to be a lipocarbohydrate.[6] Its activity is standardized in the guinea pig, and, inoculated intradermally into the wattles of turkeys, it gives the usual delayed type of hypersensitivity reaction which correlates well with the results of the complement-fixation reaction.[5]

Virus Isolation. Infection with these viruses may, of course, be established by isolation of the infectious agent from blood, sputum, or lung tissue taken at autopsy. The specimen is inoculated intranasally, intracerebrally, or intraperitoneally into mice and into the yolk sac of the embryonated egg. The virus is found as basophilic inclusion bodies and may be identified by serological methods.

Epidemiology. In the human disease the virus usually enters the body via the respiratory tract. As indicated earlier, it is most often acquired from an animal reservoir of infection, usually indirectly by inhalation of dried infectious material such as feces, and less commonly by direct contact with infected animal

Psittacine birds are the best known of these reservoirs, and infection appears to be common among wild parrots and parakeets, may assume clinical form in caged birds, and is generally endemic in aviaries and in breeding colonies of pigeons and ducks. The relative importance of mammalian, such as rodent, reservoirs of infection to the human disease is uncertain. Under some circumstances the virus may be transmitted directly from man to man as shown by a number of well-documented instances of infection of hospital or nursing personnel by patients. The highly contagious nature of the disease is evident from the number of laboratory infections which have occurred.

The incidence of the disease is largely an expression of risk. This factor accounts for its predominance in older women, and its tendency to occur as an occupational disease among professional handlers and breeders of birds. Control of the reservoir of infection in psittacine birds by quarantine has had some small success but in general depends upon avoiding sufficiently intimate contact with the animal reservoirs of endemic infection.

LYMPHOGRANULOMA VENEREUM

Lymphogranuloma venereum is a venereal disease caused by a virus closely related to the psittacosis-ornithosis-pneumonitis viruses described above. It is also known as climatic bubo, tropical bubo, lymphopathia venereum, and lymphogranuloma inguinale. The last is not to be confused with granuloma inguinale caused by *Donovania granulomatis* and discussed elsewhere (Chap. 27). It was described as a clinical entity in 1913 by Durand, Nicolas, and Favre and is sometimes known as *maladie de Nicolas et Favre,* but it was not until the 1930's that its viral etiology was recognized and the application of immunological methods allowed the grouping of various genital diseases such as elephantiasis of the female pudenda and inflammatory rectal strictures into a disease of common etiology.

The disease tends to occur in tropical regions, because of socio-economic rather than climatic conditions, but its true incidence is not known with any certainty because it is usually not reportable. In this country there appears to be a large reservoir of infection in the Negro as indicated by positive serological reactions in 25 to 40 per cent of those examined in certain areas.

The virus is morphologically indistinguishable from the others of this group and undergoes a closely similar, if not identical, growth cycle in the host cell. It is closely related immunologically through group antigens but is distinct in its pathogenicity, occurring apparently only in man as the natural host. While mice, monkeys, and embryonated eggs may be infected experimentally, this virus does not infect birds.

The Disease in Man.[20] The human disease occurs in three stages. Following the initial wide dissemination of the virus in the blood, spinal fluid, and other tissues, the primary lesion appears within a few days. It takes the form of a vesicular or herpetiform lesion occurring on the glans or prepuce of the penis, or on the posterior portions of the labia, vaginal wall, or cervix, and may also be found within the urethra and in the anal region. The vesicle breaks down to leave a shallow ulcer that is not indurated and heals without scarring. This lesion is painless and is frequently overlooked.

The second stage of the disease is an invasion of the lymphatics. The regional nodes, commonly inguinal and also pelvic in the female, become enlarged and painful to form the bubo. In half or more of cases these suppurate, resembling similar lesions found in tuberculosis and

syphilis, and may continue to drain for a long time. In this stage general as well as local symptoms occur, including fever and general aches, and in some cases arthritic and conjunctival symptoms and signs of involvement of the central nervous system may occur.

The third stage is an urethrogenitoperineal syndrome. The structural changes include nondestructive elephantiasis of the labia and clitoris in the female (esthiomene) and of the penis and scrotum in the male, and the rectum and anus become involved with the development of rectal stenosis, stricture, etc. The disease may be successfully treated with sulfonamides and the tetracyclines.[29]

Pathogenicity for Animals. The virus grows well in the yolk sac of the embryonated hen's egg. In mice inoculated intracerebrally leptomeningitis is produced, and the animals show incoordination, weakness, and paresis with a fatality rate of perhaps 30 per cent. On intranasal inoculation, the virus produces pneumonitis, often fatal within a week, which takes the form of a desquamative alveolitis with nodular inflammation around the capillaries and lymphatics, and the virus is demonstrable microscopically in the affected tissues.

The human disease is reasonably well reproduced in the monkey when the virus is inoculated into the prepuce, lymph nodes, or rectal tissue, with the development of typical inflammatory reactions. The monkey may also be infected by the intraperitoneal, intraocular, and intranasal routes, but not by intravenous or intracerebral inoculation.

Immunity. Effective immunity to this disease appears to be an infection immunity associated with continued presence of the virus. Experimental inoculation of an infected individual gives a reaction similar to the Frei test (see below) but does not lead to the appearance of the initial vesicular lesion or an inflammatory response in the regional lymphatics.

The immune response is evidenced by the appearance of complement-fixing antibody in 2 to 4 weeks and the development of a hypersensitivity to the virus substance. Both are useful for diagnostic purposes. The complement-fixation reaction is positive with serums from individuals infected with other viruses of the group, *viz.*, psittacosis, etc., but may be made more specific by preliminary adsorption of the serum with heterologous virus, and syphilitic serums may be nonspecifically positive. It is often not possible to obtain serum early in the disease, but a titer of 1:32 in conjunction with typical clinical findings is regarded as diagnostic, and an increasing titer has greater value.

Hypersensitivity to the virus substance was observed by Frei in 1925, and is demonstrable as a typical delayed reaction following intradermal inoculation of virus. It was, in fact, the application of this reaction, the Frei test, that led to the identification of apparently diverse genital diseases, which had been known for many years, as manifestations of infection with this virus. The hypersensitivity develops 1 to 6 weeks after infection and remains positive apparently for the life of the individual, but transitory negative reactions may occur during sulfonamide therapy. The original Frei antigen was a 1:5 dilution of pus from a bubo in saline sterilized by heating. Mouse brain antigens have not been satisfactory because of nonspecific reactions, and antigen prepared from the yolk sac of the infected egg is generally used. The skin test antigen is marketed under the name Lygranum. Cross-reactions[33] occur, but an acid extract of the virus appears to be more specific.

Epidemiology. The disease occurs all over the world[15] and is found largely in ignorant persons of low economic status. It is usually, though not necessarily, transmitted by sexual intercourse. Infection of the eye with an oculoglandular syndrome is known, and primary infection may occur in the mouth with painless vesicular lesions and swelling of the tongue, followed by infection of the glands of the neck. Occasional acci-

dental infections occur, as in hospital orderlies infected while cleaning patients and in surgeons infected during removal of infected lymph glands. Control of the disease is difficult because it is not ordinarily reportable.

TRACHOMA AND INCLUSION CONJUNCTIVITIS[9, 55]

Trachoma and inclusion conjunctivitis are similar diseases of the external eye which are caused by viruses related to those of the psittacosis-lymphogranuloma venereum group. Naturally occurring infections are found only in man, and the host specificity is sharp in experimental infections which are similar to, but generally milder than, those in man, and can be produced only in certain primates.

TRACHOMA[52, 56, 60, 61]

Trachoma is an ancient disease and is referred to in the earliest known writings. It occurs in the Mediterranean area, especially in North Africa and the Middle East, in Russia, and the Orient, and in this country is found among mountain people in Tennessee, Kentucky, and West Virginia and among Indians on reservations. Its incidence is greatest, to as much as 90 per cent, in Egypt and the Middle East.

The Virus. There has been evidence of the viral etiology of trachoma, and inclusion blennorrhea, since the work of the Trachoma Commission in the early 1930's. The reported isolation of trachoma virus in the embryonated egg by Tang et al.[57] in 1957 was subsequently confirmed by isolations in Africa,[13, 58] in Taiwan and in this country.[30] The virus may be grown in cultures of human amnion cells, the McCoy cell line derived from human synovial tissue,[50] and some strains may be adapted to HeLa cells,[19] as well as in the yolk sac of the embryonated egg. In its morphology and other growth characteristics it resembles closely the other viruses of this group.[28, 51] It is related antigenically through group antigen,[8] but when washed elementary bodies are used as complement-fixing antigen, the reaction is reported to be specific.[65] The elementary bodies contain a toxin, but it may not be antigenically homogeneous.[4] The culture virus is susceptible to the broad spectrum antibiotics[59] and reproduces the disease in various primates[12, 18] with strain differences in virulence.

Disease in Man. When the disease develops slowly, the first sign of infection is a slight ptosis of the lids, and there is a follicular hypertrophy of the upper tarsal conjunctiva. When the disease develops rapidly in a fulminating form, there is an inflammatory reaction characterized by a papillary or follicular hypertrophy of the conjunctiva and a mucopurulent exudate, and secondary bacterial infection is common, occurring in 50 per cent or more of cases. The exudate is polymorphonuclear in contrast with the exudates of other conjunctival infections of virus etiology, such as epidemic keratoconjunctivitis and herpes simplex infection of the eye, which are characteristically mononuclear. Intracytoplasmic inclusion bodies are found in the conjunctival and corneal epithelial cells in expressed follicular material and are most numerous in the cells from the superficial layers of epithelium from the upper tarsal and upper limbus areas.[62]

The disease progresses with vascularization of the cornea and pannus formation and secondary cicatrization of the cornea, and partial or total blindness may result. It may be successfully treated with sulfonamides or the broad spectrum antibiotics.[53]

There appears to be little or no effective immunity since reinfection or relapse is not uncommon. Infected indi-

viduals are negative to the Frei test but develop antibody which fixes complement in the presence of psittacosis and related antigens. The complement-fixation test is not sufficiently specific for diagnostic purposes, *i.e.*, it does not distinguish between trachoma and inclusion conjunctivitis.

INCLUSION CONJUNCTIVITIS

Inclusion conjunctivitis (inclusion blennorrhea, swimming pool conjunctivitis) is a benign conjunctivitis found in the newborn and in adults. It differs from trachoma in that pannus and scarring of the cornea are not observed, and the disease is self-limiting and persistent chronic infections do not seem to occur although it may persist for some months, or even as long as a year in the adult.

A virus has been isolated in the yolk sac of embryonated eggs by blind passage and eventual (9th passage) adaptation to the egg.[31] The culture virus produces a mucopurulent conjunctivitis in cynomolgus monkeys, and does not affect the cornea. It may be grown in cell culture[11] and appears to be closely related in all respects to the trachoma viruses. A preliminary study[10] has suggested that an immunity effective under experimental conditions may be produced by vaccine.

Disease in Man. In the newborn infant the incubation period is 5 to 12 days. The onset of the disease is sudden and characterized by an acute infiltration of the conjunctiva of the lower lid and a purulent exudate. The round cell infiltration results in a thickening of the conjunctiva, and the epithelium is infiltrated with polymorphonuclear cells and contains basophilic intracytoplasmic inclusion bodies indistinguishable from those of trachoma. The demonstration of these bodies, in epithelial scrapings rather than exudate films taken for examination for bacteria, is diagnostic. Occasionally the disease is severe with the formation of transient pseudomembranes, but ordinarily it may be differentiated from gonorrheal ophthalmia on clinical grounds. The acute stage persists for perhaps 2 weeks and then gradually subsides, but the cornea does not return to normal for some months and may show evidence of infiltration for as long as a year.

The disease in the adult differs in that it is an acute follicular conjunctivitis with little discharge and a mild periauricular adenopathy. The follicles have the same appearance as those found in trachoma, but the hypertrophy is more marked in the lower lid, there is an absence of corneal change, and, on microscopic examination, the follicular material does not show necrotic changes. It may, however, be difficult to differentiate clinically from other forms of acute follicular conjunctivitis, and clinical diagnosis must be confirmed by the presence of inclusion bodies. The adult disease is also self-limited, resolving spontaneously without residual corneal or conjunctival changes, but tends to persist longer than in the infant.

Although overtly a disease of the eye, the infection is, in fact, one of the genitourinary tract and as such is transmitted venereally. Inclusion bodies are found in scrapings from the genitourinary tract of mothers of diseased infants, and the infection is apparently limited to the external os of the cervix and to transitional epithelium histologically closely similar to conjunctival epithelium. The infection is asymptomatic in the female but often produces a nonspecific urethritis in the male. Although of minor significance clinically, the genitourinary infection may be widespread.

The infant acquires the disease at birth from the infected mother, while the adult disease is most often acquired in swimming pools in which the water, which is not subjected to adequate chlorination or other treatment, is infected by persons having the genitourinary infection. Consistent with the nature of the reservoir of infection, inclusion conjunctivitis is an occupational disease of obstetricians and gynecologists. The serum of infected persons gives positive complement-fixation reac-

tions with the group antigens and is not diagnostic for this disease. The disease in infants is not prevented by prophylactic silver nitrate, but both the infant and adult disease may be treated successfully with sulfonamides or tetracyclines, usually applied topically.

REFERENCES

1. Allen, E. G., and Bovarnick, M. R.: Association of reduced diphosphopyridine nucleotide cytochrome C reductase activity with meningopneumonitis virus. J. Exptl. Med., 1957, **105:** 539–547.
2. Bader, J. P., and Morgan, H. R.: Latent viral infection of cells in tissue culture. VI. Role of amino acids, glutamine and glucose in psittacosis virus propagation in L cells. J. Exptl. Med., 1958, **108:**617–630.
3. Bedson, S. P.: The psittacosis-lymphogranuloma group of infective agents. J. Roy. Inst. Pub. Health Hyg., 1959, **22:**67–78; 99–112; 131–143.
4. Bell, S. D., Jr., Snyder, J. C., and Murray, E. S.: Immunization of mice against toxic doses of homologous elementary bodies of trachoma. Science, 1959, **130:**626–627.
5. Benedict, A. A., and McFarland, C.: Newer methods for detection of avian ornithosis. Ann. N. Y. Acad. Sci., 1958, **70:**501–515.
6. Benedict, A. A., and O'Brien, E.: Antigenic studies on the psittacosis-lymphogranuloma venereum group of viruses. II. Characterization of complement-fixing antigens extracted with sodium lauryl sulfate. J. Immunol., 1956, **76:**293–300.
7. Benedict, A. A., and O'Brien, E.: A passive hemagglutination reaction for psittacosis. J. Immunol., 1958, **80:**94–99.
8. Bietti, G. B., Babudieri, B., and Pannarale, M. R.: Existence of antigenic affinity between the agents of trachoma and those of venereal lymphogranuloma-psittacosis. Bull. W. H. O., 1957, **16:**1041–1043.
9. Collier, L. H.: On the aetiology and relationship of trachoma and inclusion blennorrhoea. Rev. Internat. Trachome, 1960, **37:**585–599.
10. Collier, L. H.: Experiments with trachoma vaccines. Experimental system using inclusion blennorrhoea virus. Lancet, 1961, **i:**795–800.
11. Collier, L. H.: Growth characteristics of inclusion blennorrhea virus in cell cultures. Ann. N. Y. Acad. Sci., 1962, **98:**42–49.
12. Collier, L. H.: Experimental infection of baboons with inclusion blennorrhea and trachoma. Ann. N. Y. Acad. Sci., 1962, **98:**188–196.
13. Collier, L. H., and Sowa, J.: Isolation of trachoma virus in embryonate eggs. Lancet, 1958, **i:**993–996.
14. Colon, J.: The role of folic acid in the metabolism of members of the psittacosis group of microorganisms. Ann. N. Y. Acad. Sci., 1962, **98:**234–249.
15. Coutts, W. E.: Lymphogranuloma venereum; general review. Bull. W. H. O., 1950, **2:**545–562.
16. Cox, H. R.: Psittacosis, ornithosis and related viruses. Ann. N. Y. Acad. Sci., 1947, **48:**393–414.
17. Cox, H. R.: Viral and rickettsial toxins. Ann. Rev. Microbiol., 1953, **7:**197–218.
18. Dawson, C. R., Mordhorst, C. H., and Thygeson, P.: Infection of rhesus and cynomolgus monkeys with egg-grown viruses of trachoma and inclusion conjunctivitis. Ann. N. Y. Acad. Sci., 1962, **98:**167–176.
19. Furness, G., Graham, D., Reeve, P., and Collier, L. H.: The growth of trachoma and inclusion blennorrhoea viruses in cell culture. Rev. Internat. Trachome, 1960, **37:**574–584.
20. Galbraith, H. J. B., Graham-Stewart, C. W., and Nicol, C. S.: Lymphogranuloma venereum. Brit. Med. J., 1957, **ii:**1402–1405.
21. Gildemeister, H., et al.: Praktikum der Virusdiagnostik. Gustav Fischer Verlag, Stuttgart. 1961.
22. Gogolak, F. M.: The mouse erythrocyte hemagglutinin of feline pneumonitis virus. J. Infect. Dis., 1954, **95:**220–225.
23. Gogolak, F. M., and Ross, M. S.: The properties and chemical of the psittacosis virus hemagglutinin. Virology, 1955, **1:**474–496.
24. Gogolak, F. M., and Weiss, E.: The effect of antibiotics on agents of the psittacosis-lymphogranuloma group. J. Infect. Dis., 1950, **87:**264–274.
25. Gordon, F. B., Andrew, V. W., and Wagner, J. C.: Development of resistance to penicillin and to chlortetracycline in psittacosis virus. Virology, 1957, **4:**156–171.
26. Gordon, F. B., and Mamay, H. K.: Combination of characters (drug resistance) in a single strain of psittacosis virus. Science, 1957, **126:** 354–355.
27. Gordon, F. B., and Quan, A. L.: Drug susceptibilities of the psittacosis and trachoma agents. Ann. N. Y. Acad. Sci., 1962, **98:**261–274.
28. Gordon, F. B., Quan, A. L., and Trimmer, R. W.: Morphologic observations on trachoma virus in cell cultures. Science, 1960, **131:**733–734.
29. Greaves, A. B., et al.: Chemotherapy in bubonic lymphogranuloma venereum. A clinical and serological evaluation. Bull. W. H. O., 1957, **16:**277–289.
30. Hanna, L.: Isolation of trachoma and inclusion conjunctivitis viruses in the United States. Ann. N. Y. Acad. Sci., 1962, **98:**24–30.
31. Hanna, L., et al.: Virus isolated from inclusion conjunctivitis of newborn (inclusion blennorrhea). Science, 1960, **132:**1660–1661.
32. Higashi, N., Tamura, A., and Iwanaga, M.: Developmental cycle and reproduction mechanism of the meningopneumonitis virus in strain L cells. Ann. N. Y. Acad. Sci., 1962, **98:** 100–121.
33. Hilleman, M. R., Greaves, A. B., and Werner, J. H.: Group-specificity of psittacosis–lymphogranuloma venereum group. Skin test antigens in lymphogranuloma patients. J. Lab. Clin. Med., 1958, **52:**53–57.
34. Hilleman, M. R., Haig, D. A., and Helmold, R. J.: In vivo and in vitro studies of serological specificity among viruses of the psittacosis-lymphogranuloma venereum group. J. Immunol., 1952, **68:**121–129.
35. Holtermann, O. A., Mergenhagen, S. E., and Morgan, H. R.: Factors related to psittacosis virus (strain 6BC) growth. V. Folic acid-like factors in infected cells. Proc. Soc. Exptl. Biol. Med., 1959, **100:**370–372.

36. Hurst, E. W.: Chemotherapy of virus diseases. Brit. Med. Bull., 1953, **9:**180–185.
37. Jenkin, H. M.: Preparation and properties of cell walls of the agent of meningopneumonitis. J. Bact., 1960, **80:**639–647.
38. Jenkin, H. M., Ross, M. R., and Moulder, J. W.: Species-specific antigens from the cell walls of the agents of meningopneumonitis and feline pneumonitis. J. Immunol., 1961, **86:**123–127.
39. Karrer, H., Meyer, K. F., and Eddie, B.: The complement fixation inhibition test and its application to the diagnosis of ornithosis in chickens and in ducks. J. Infect. Dis., 1950, **87:**13–23.
40. Litwin, J.: The growth cycle of the psittacosis group of microorganisms. J. Infect. Dis., 1959, **105:**129–160.
41. Manire, G. P., and Meyer, K. F.: The toxins of the psittacosis-lymphogranuloma group of agents. I. The toxicity of various members of the psittacosis lymphogranuloma venereum group. J. Infect. Dis., 1950, **86:**226–232.
42. Manire, G. P., and Meyer, K. F.: The toxins of the psittacosis-lymphogranuloma group of agents. III. Differentiation of strains by the toxin neutralization test. J. Infect. Dis., 1950, **86:**241–250.
43. Meyer, K. F.: The natural history of plague and psittacosis. Pub. Health Repts., 1957, **72:**705–719.
44. Morgan, H. R.: Factors related to the growth of psittacosis virus (strain 6BC). IV. Certain amino acids, vitamins and other substances. J. Exptl. Med., 1954, **99:**451–460.
45. Morgan, H. R., and Bader, J. P.: Latent viral infection of cells in tissue culture. IV. Latent infection of L cells with psittacosis virus. J. Exptl. Med., 1957, **106:**39–44.
46. Moulder, J. W.: Biochemical aspects of the growth of feline pneumonitis virus with chick embryo yolk sac. Bact. Rev., 1954, **18:**170–176.
47. Moulder, J. W.: Structure and chemical composition of isolated particles. Ann. N. Y. Acad. Sci., 1962, **98:**92–99.
48. Moulder, J. W., et al.: Production and properties of a penicillin-resistant strain of feline pneumonitis virus. J. Infect. Dis., 1955, **96:**57–74.
49. Moulder, J. W., Ruda, J., Colon, J. I., and Greenland, R. M.: The effect of passage with chloramphenicol upon the behavior of penicillin-resistant feline pneumonitis virus during subsequent passage with penicillin. J. Infect. Dis., 1959, **102:**186–201.
50. Pollard, M., Starr, T. J., Tanami, Y., and Moore, R. W.: Propagation of trachoma virus in cultures of human tissues. Proc. Soc. Exptl. Biol. Med., 1960, **104:**223–225.
51. Pollard, M., and Tanami, Y.: Cytochemistry of trachoma virus replication in tissue cultures. Ann. N. Y. Acad. Sci., 1962, **98:**50–61.
52. Public Health Service: Trachoma manual and atlas. Public Health Service Publication No. 541. U. S. Department of Health, Education and Welfare, 1958.
53. Report: Expert Committee on Trachoma—second report. W. H. O. Technical Report No. 106. World Health Organization, Palais des Nations, Geneva. 1956.
54. Ross, M. R., and Jenkin, H. M.: Cell wall antigens from members of the psittacosis group of organisms. Ann. N. Y. Acad. Sci., 1962, **98:**329–336.
55. Siniscal, A.: Trachoma story. Pub. Health Repts., 1955, **70:**497–507.
56. Symposium: The biology of the trachoma agent. Ann. N. Y. Acad. Sci., 1962, **98:**1–382.
57. Tang, F.-F., Huang, Y.-T., Chang, H.-L., and Wong, K.-C.: Isolation of trachoma virus in chick embryo. J. Hyg. Epidemiol. Microbiol. Immunol., 1957, **1:**109–120.
58. Tarizzo, M. L.: Études sur le trachome. I. Isolement et culture en série de 12 souches de virus sur oeuf embryonné. Bull. W. H. O., 1961, **24:**103–105.
59. Tarizzo, M. L., Nataf, R., and Daghfous, T.: Études sur le trachome. 2. Action des antibiotiques sur le virus du trachome cultivé dans les oeufs embryonnés. Bull. W. H. O., 1961, **24:**107–113.
60. Thygeson, P.: Trachoma virus: Historical background and review of isolates. Ann. N. Y. Acad. Sci., 1962, **98:**6–13.
61. Thygeson, P., and Nataf, R.: Aetiology of trachoma. Rev. Internat. Trachome, 1958, **35:**83–175.
62. Thygeson, P., and Nataf, R.: Etiologic problems in trachoma. Rev. Internat. Trachome, 1958, **35:**177–227.
63. Weiss, E.: The nature of the psittacosis-lymphogranuloma group of microorganisms. Ann. Rev. Microbiol., 1955, **9:**227–252.
64. Wenner, H. A.: Psittacosis-lymphogranuloma group of viruses. Adv. Virus Res., 1958, **5:**39–93.
65. Woolridge, R. L., Jackson, E. B., and Grayston, J. T.: Serological relationship of trachoma, psittacosis and lymphogranuloma venereum viruses. Proc. Soc. Exptl. Biol. Med., 1960, **104:**298–301.

Chapter 37

THE VIRUSES OF EXANTHEMATOUS DISEASE AND THE TUMOR VIRUSES

A NUMBER of viral diseases of man and of lower animals are exanthematous in nature, and the viruses causing them have been said to be dermotropic because the predominant lesions occur in the skin. Certain of these make up the more or less homogeneous group of pox viruses, having in common the properties of relatively large size, often just within the limits of optical resolution, and a brick shape, and occurring as intracytoplasmic inclusion bodies in the infected cells. Others, however, are spheroidal in shape, range in size from somewhat below the limits of optical resolution to one of the smallest of the known viruses, that of foot-and-mouth disease, and infected cells may contain intracytoplasmic or intranuclear inclusion bodies. They are heterogeneous and, with minor exceptions, as varieties of herpes virus, do not fall into groups.

While infection of the host cell with a virus usually results in relatively rapid death, a number of these viruses induce a proliferative response which may be relatively brief and followed by necrotic changes or sufficiently prolonged that hyperplasia is an outstanding feature of the disease. The proliferative response is apparent, though transitory, in smallpox, is more pronounced in molluscum contagiosum in man and in fibroma in rabbits, and in rabbit papilloma and myxoma the process is so marked that the virus appears to have carcinogenic properties. The viruses which induce a pronounced proliferative response are sometimes grouped as the tumor viruses; such a group is heterogeneous; *e.g.*, it includes both pox-like and smaller spheroid viruses.

THE POX GROUP OF VIRUSES[62, 68]

The viruses making up the pox group resemble one another morphologically and in the pathogenesis of the diseases they produce, and they are immunologically related to one another. They are differentiated by host specificity and relative pathogenicity. The group includes the virus of variola which occurs in man and that of vaccinia, together with the viruses causing a group of pox diseases of lower animals. The varicella-zoster viruses of man are considered here although they resemble the herpes viruses in forming intranuclear inclu-

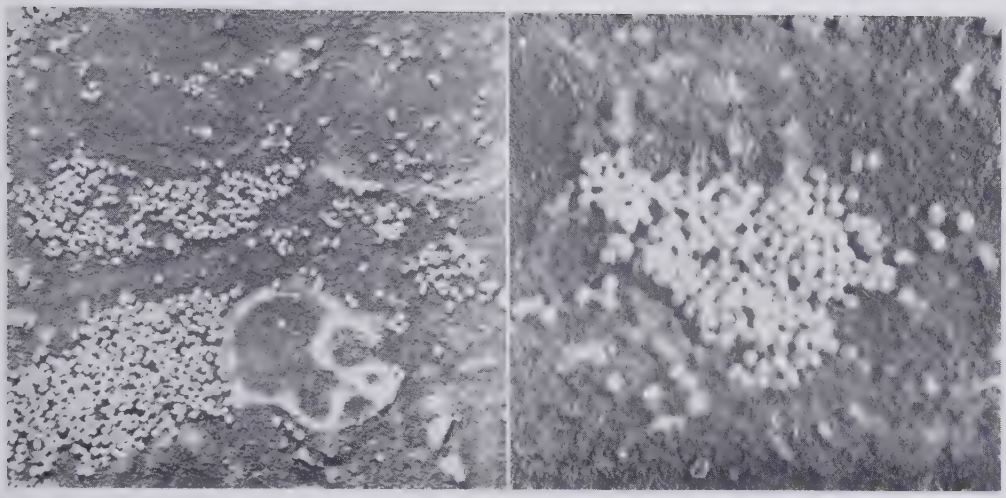


Figure 265. Electron micrograph of thin section through the chorioallantoic membrane of a 10-day chicken embryo 24 hours after inoculation with vaccinia. The numerous elementary bodies can be seen to possess a cuboidal or cylindrical shape. *Left,* × 3000; *right,* × 8300. (Wyckoff, Zeitsch. f. Zellforsch.)

sions. The viruses of molluscum contagiosum and of rabbit fibroma and myxoma are closely similar morphologically but tend to be set apart from the pox group on the basis of the diseases they produce. They are considered with the tumor viruses.

Morphology. The viruses of this group are among the largest of the viruses, and the elementary body is just within the limits of optical resolution. The higher resolution made possible by electron microscopy has made it clear that, at least in many preparations suitable for examination by this method, the virus particle is a regular six-sided structure with rounded corners often described as brick-shaped, with dimensions of 200 to 250 by 250 to 350 mμ. In some preparations the bodies appear to be ovoid in shape and in either case contain a central mass of electron-dense material, presumably nucleic acid.

These viruses occur as intracytoplasmic inclusion bodies known as Guarnieri bodies in the case of variola and vaccinia and as Bollinger bodies in fowlpox. These bodies differentiate into, and in mature form are composed of, elementary bodies called the Paschen bodies of variola and vaccinia and Borrel bodies of fowlpox. Both inclusion bodies and elementary bodies may be stained with polychrome stains such as Giemsa, by steaming with bicarbonate–crystal violet, and other methods, but do not stain satisfactorily with the usual bacterial stains.

Chemical composition. Like the rickettsiae and the large viruses of the psittacosis-lymphogranuloma venereum group, the chemical composition of the pox viruses is similar to that of bacteria, including nucleic acid in amounts of 6 to 7 per cent in contrast with the large amounts found in some other viruses, together with protein, polysaccharide, and lipid. Highly purified preparations have been found to contain copper and flavin together with biotin. If these are to be regarded as integral parts of the virus, they may represent rudimentary or vestigial fragments of a respiratory mechanism, but it is at least equally possible that such substances represent contamination originating in the infected tissue.

Growth. Viral replication appears to occur in the cytoplasm of the infected cell. Following entry of the virus particle into the host cell, a portion of the cytoplasm surrounding it differentiates to form a matrix. When the matrix is dense it is the inclusion body, but viral replication may occur in a matrix that is not sufficiently dense to appear as an in-

clusion body. The matrix subsequently breaks down into elementary bodies, which may be in various developmental stages as described elsewhere (Chap. 4), and eventually the mass breaks down completely, and the cytoplasm of the cell is filled with elementary bodies which may then infect other cells.

Antigenicity. At least five kinds of antigens are present in the viruses of this group, certain of which are much better known in some of the viruses than in others. The LS soluble antigen of variola and vaccinia, found free in infected tissues, appears to be a single complex of which the L antigen is heat-labile and the S antigen is heat-stable. These antigens are demonstrable by complement fixation. A nucleoprotein (NP) antigen is present also, and these viruses form a hemagglutinin which agglutinates fowl erythrocytes, and in the case of mousepox mouse erythrocytes also, which is separable from the elementary body and associated with particles about 65 mμ in diameter. The hemagglutinin activity is specifically inhibited in the presence of antibody.

During the replication cycle of the virus in HeLa cell culture these antigens appear in sequence. The LS antigen is detectable in about 4 hours, and the NP antigen in 5–6 hours, both in the cytoplasm of the infected cell and preceding the appearance of the infective particle. The hemagglutinating antigen is apparently a by-product of virus-cell interaction and is not found until about 10 hours post infection.[81]

None of the antibodies to these antigens is associated with neutralization or protection; rather, the ability to stimulate protective antibody formation is associated with infectivity of the virus strain, and it appears to involve antigens other than those just described.[4]

Pathogenesis of Pox Disease. Although there is a considerable degree of host specificity among these viruses, differences in the pathogenesis of the diseases they produce may be considered to be variations on the same theme. The proliferative response alluded to above, for example, is minor in variola and vaccinia, but in some forms of fowlpox so marked that the disease has been known as contagious epithelioma.

The viruses may be grouped on the basis of tissue tropisms, *viz.*, the pox viruses are dermotropic, and such tropisms are inferred from the site of the overt or predominant lesion. It is significant, however, that viral replication may occur without obvious damage in cells and tissues apart from those in which the predominant lesions occur, and that

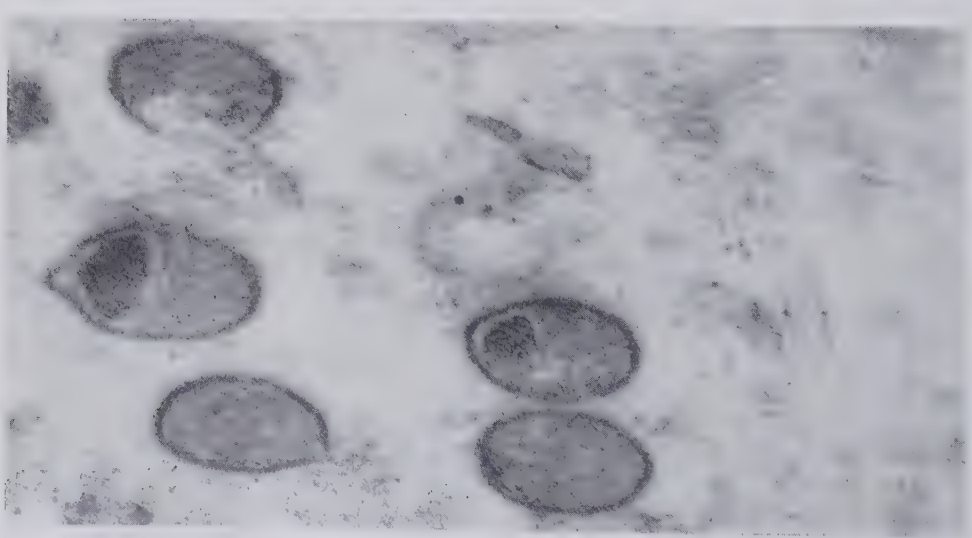


Figure 266. Electron micrograph of vaccinia virus in a section of infected chorioallantoic membrane showing the oval shape observed in some preparations. Note the small mass of electron-dense material in two of the virus particles. × 63,500. (Morgan, J. Exptl. Med.)

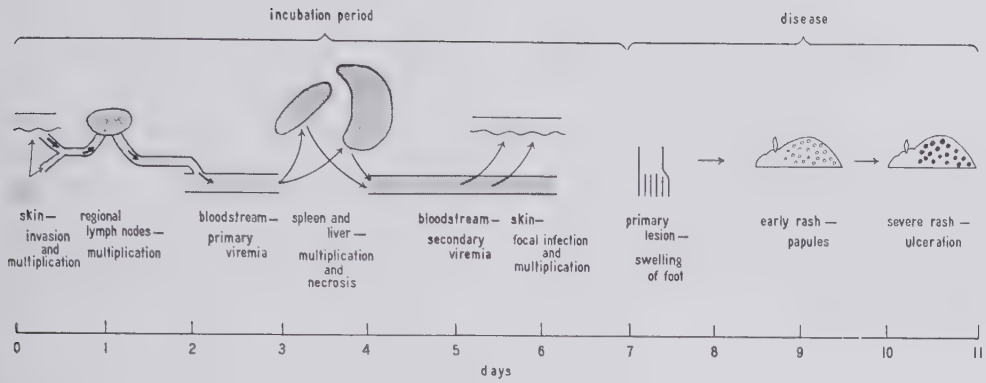

incubation period
disease
skin— invasion and multiplication
regional lymph nodes— multiplication
bloodstream— primary viremia
spleen and liver— multiplication and necrosis
bloodstream— secondary viremia
skin— focal infection and multiplication
primary lesion— swelling of foot
early rash — papules
severe rash — ulceration
0 1 2 3 4 5 6 7 8 9 10 11
days

Figure 267. Diagrammatic representation of the pathogenesis of mousepox infection. (Redrawn from Fenner.)

such replication and dissemination may be an integral part of the pathogenesis of the disease. The occurrence of viremia, for example, exposes the virus to the action of humoral antibody and is fundamental to effective immunity to such a disease. In the case of mousepox the sequence of events has been worked out in detail by Fenner[44] and it is a fair presumption that this disease may be taken as a prototype, for the present at least, of similar pox diseases such as variola and vaccinia.

The incubation period of mousepox is about 7 days, and by 11 days after infection the disease has progressed to severe rash with ulceration. Following initial infection, the virus multiplies in the skin and spreads to the regional lymphatics and multiplies there within the first 2 days of the incubation period. The virus is then shed into the blood stream to give primary viremia in the second or third day, spreading the infection to the spleen and liver where it multiplies further and produces necrosis. Virus is again shed into the bloodstream to give secondary viremia on the fourth and fifth days, spreading the infection to the skin to set up foci of infection on the sixth day. By the seventh day, the end of the incubation period, the primary lesion, a swelling of the footpads, occurs; a papular rash appears by the ninth day which progresses to severe rash and ulceration by the eleventh day. This sequence of events is illustrated diagrammatically in the accompanying figure. Clearly, during the incubation period the virus is widely disseminated in the body, multiplying in cells not concerned in the primary lesion of the disease. As will appear (Chap. 39), analogous phenomena occur in infections with other kinds of viruses also.

VARIOLA AND VACCINIA

Smallpox (variola) is an ancient disease which is known to have occurred in epidemic form in China as early as the twelfth century B.C., and is present in endemic, and from time to time epidemic, form in Asia, Africa, and the Middle East. It was widely disseminated in Europe during the Crusades, and was apparently introduced into the Western Hemisphere in the early sixteenth century, spreading into Central and South America, and new infection continued to be introduced by the slave trade.

This disease occurs in two forms differing markedly in severity. The one, variola major (malignant smallpox, black smallpox, etc.), is characterized by a case fatality rate of 25 to as much as 40 per cent. The milder form, variola minor, has a case fatality rate of perhaps 1 per cent, and has been given a variety of names including alastrim, Kaffir pox, cotton pox, Cuban itch, paravariola, etc. The causative viruses of the two forms of the disease are almost indistinguishable except for a slightly greater viru-

lence of variola major for the chick embryo. This difference in virulence may be enhanced by varying the temperature of incubation to give a laboratory test.[39, 98] The diseases are differentiated on epidemiological grounds, *i.e.*, on the basis of the case fatality rate.

In many areas, including the United States, variola minor has tended to displace the malignant form of the disease. At the present time Asia and Africa are the main centers of variola major infection, with India the greatest single source of cases, and this form of the disease is endemic in Portugal in Europe and in Mexico in North America.[97] The introduction of variola major from endemic centers of infection remains a constant possibility; for example, this occurred in New York City in 1947. In this country the number of cases of smallpox has declined from 30,000 to 50,000 cases reported per year in the 1920's to no confirmed cases being reported since 1954.

Vaccinia is almost always a purposefully acquired infection to produce immunity to smallpox (see below) and its occurrence in this way dates from Jenner's observations in the latter part of the eighteenth century. Vaccinia is almost always a mild, localized disease in man; generalized vaccinia occurs rarely in the normal individual, but may occur when a nonimmune child with previously existing skin lesions, *e.g.*, eczema, acquires vaccinia by contact.

Smallpox in Man. Variola virus is resistant to drying, and the crusts from pustules are highly infectious. Human infection is acquired by contact, direct or indirect, with such infectious material. There is reason to believe[37] that in the typical disease primary infection occurs in the respiratory tract with multiplication of the virus in minimal, often but not necessarily noninfectious, lesions.[38] The virus spreads to the regional lymphatics, and into the blood stream to the viscera where it multiplies during the latter part of the incubation period of about 12 days. When virus is released from these sites to give secondary viremia, symptoms, including chills, fever, prostration, headache, backache, and vomiting, appear, and the virus may be found in the blood. The secondary viremia gives rise to foci of infection in the skin, mucous membranes, and viscera, and the skin lesions appear. These occur as a single crop and are at first macular, and then pass through papular and vesicular stages to the pustule over a period of 5 to 10 days.

Histologically there is proliferation in the prickle-cell layer of the skin with the appearance of cytoplasmic inclusions. Intracellular edema, evidenced by the

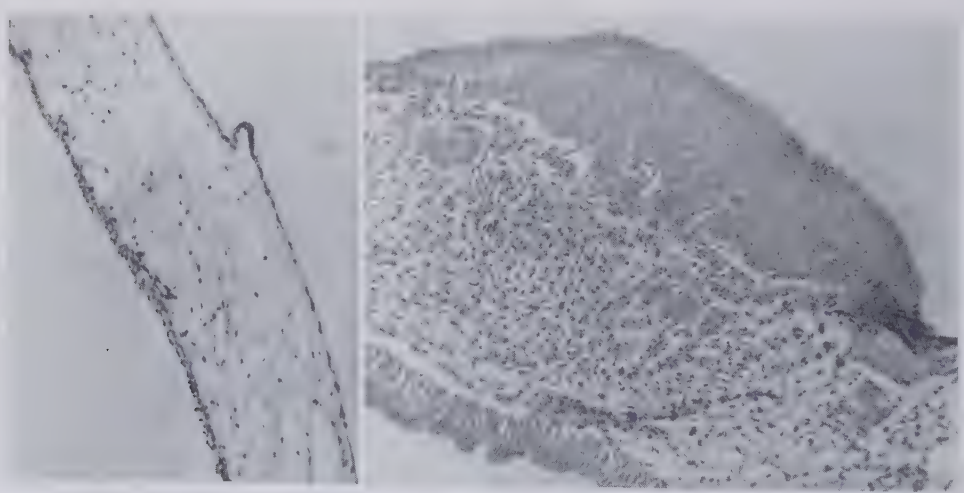


Figure 268. Sections of chorioallantoic membranes of 13-day embryonated eggs. *Left*, uninoculated; *right*, section through a variola lesion. Note the thickening of the membrane and discrete area of epithelial hyperplasia. × 100. (U. S. Army photograph, courtesy of Dr. N. Hahon.)

appearance of vacuoles, occurs and is followed by intercellular edema. The vacuoles enlarge, become confluent, the cell membrane is distended and breaks down, and vesicles are formed by the coalescence of adjacent affected cells. The epidermal reaction is followed by dilatation of blood and lymph vessels, involvement of the basilar layer, the appearance of an inflammatory exudate, and necrosis in the corium which leads to the typical scarring.

The most common complication is pyogenic infection, and antibiotic therapy is effective in minimizing such infection with consequent reduction in residual scarring, although chemotherapeutic agents are ineffective against the virus infection.

Immunity. Smallpox has been the classical example of infectious disease in which recovery is associated with the development of a highly effective and long-lasting immunity, and there is no doubt that the individual remains immune for many years. The immune response is evident in the appearance, during the course of the disease, of complement-fixing and hemagglutinin-inhibiting antibody activity, but, as indicated earlier, these antibodies do not appear to be associated with effective immunity. Protective antibody is formed which, unlike complement-fixing and hemagglutinin-inhibiting antibodies that decline to insignificant levels in some 12 months, is demonstrable for years, and may be demonstrated by appropriate tests such as tissue culture assay.[27, 86]

There is a high degree of cross-immunity among the various kinds of pox viruses which is demonstrable in experimental animals. That between variola and vaccinia is of a high order and allows the use of the latter mild infection to produce an effective prophylactic immunity to smallpox in man (see below).

Laboratory diagnosis. The laboratory diagnosis of smallpox assumes significance in the prompt and accurate identification of atypical cases in which the disease is modified by prior vaccination, and to exclude generalized vaccinia, varicella (see below), etc. It is dependent upon the demonstration of the presence of the virus, either directly or inferentially from the presence of specific antibody.

Inclusion bodies are found in stained smears from lesions in the papular and vesicular stages of the disease, and virus may be isolated from these lesions on the chorioallantoic membrane of the 12 to 14 day old embryonated hen's egg. Specific antigen is demonstrable in the lesions by complement fixation in the presence of known antiserum.

The most readily applicable diag-

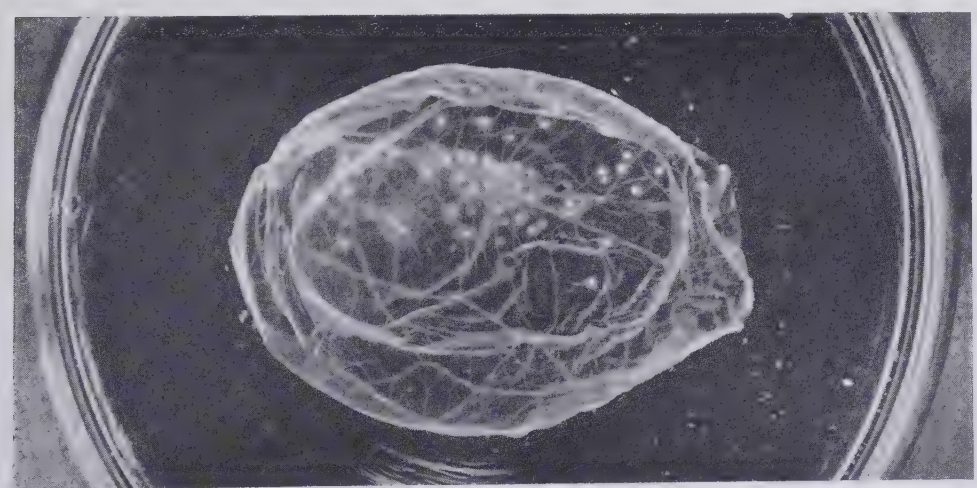


Figure 269. The gross appearance of the pock-like lesions produced by variola virus on the chorioallantoic membrane of the embryonated egg after 72 hours' incubation. Hematoxylin and eosin; × 100. (Hahon and Ratner, J. Bact.)

nostic tests are those for serum antibody, complement-fixing antibody, and hemagglutinin-inhibiting antibody. The former may not appear until the later eruptive stage of the disease. It has been reported[24] that hemagglutinin-inhibiting antibody is detectable even in the preeruptive stage of the disease, reaching titers of 1:2000 by the end of the first week. Antibody titer is of questionable diagnostic significance in recently vaccinated persons, but a rising titer during the course of the disease may be regarded as diagnostic.

Vaccinia and Immunization. Antibody to the LS and NP antigens is produced by inoculation with noninfectious material, but it has not been possible to produce an effective immunity to smallpox by the use of inactive virus. The development of such an immunity appears to be dependent upon infection with variola or related viruses of which vaccinia is the most important.

The practice of variolation, deliberate inoculation of man by application of the dried crusts of variola pustules to the skin, nasal mucous membranes, or by ingestion, was common in the Orient in ancient times. It was introduced into Europe in 1718 by Lady Mary Wortley Montagu, wife of the British ambassador at Constantinople. The infection so produced was usually much less severe than naturally acquired smallpox, and the appreciable mortality rate considerably less than that of the natural disease.

Meanwhile it had been observed by a number of persons that those who contracted cowpox from infected cattle did not acquire smallpox upon subsequent exposure. Cowpox is an exceedingly mild disease in man, rarely if ever fatal, in which the lesion is almost always limited to the site of inoculation. These casual observations were extended by the Englishman Jenner who published his classic treatise on the relation of the two diseases in 1796. Prophylaxis of smallpox by inoculation with cowpox, or so-called Jennerian prophylaxis, was favorably received and widely applied.

Vaccinia virus. It was assumed by Jenner that cowpox virus is variola virus adapted to the bovine host. The cow may be infected with variola, at first with insignificant lesions following intracutaneous inoculation, but on passage in this animal a vesiculopustular eruption is produced which is similar to, but not identical with, that of naturally occurring cowpox. While variola, vaccinia, cowpox, and mousepox are closely related immunologically, antigenic differences may be distinguished by cross-neutralization tests. In this respect vaccinia is found to be more closely related to variola than to cowpox, and in turn cowpox is more closely related to mousepox than to variola.[46] This suggests that present strains of vaccinia virus were derived from variola virus rather than cowpox virus, but it has not been possible to derive them experimentally. In this connection it is of interest that naturally acquired cowpox in man is a somewhat more severe disease than vaccinia, and that in recombination experiments with vaccinia variants in mixed infections in HeLa cell culture and on the chorioallantoic membrane, the recombinants, apparently analogous

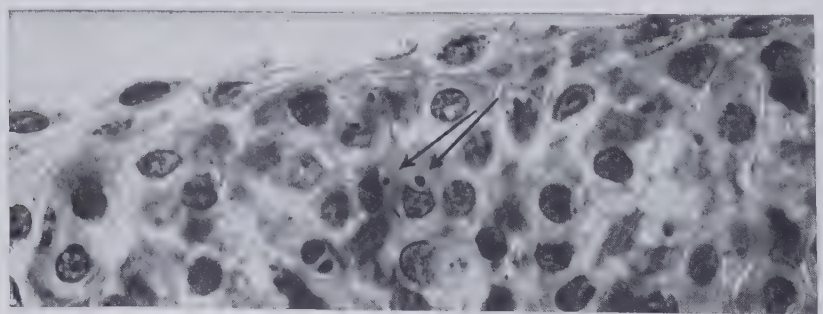


Figure 270. Guarnieri bodies, the intracytoplasmic inclusion bodies of vaccinia. The arrows point to two inclusions among many in the infected corneal epithelium of a rabbit's eye. Giemsa stain; × 650.

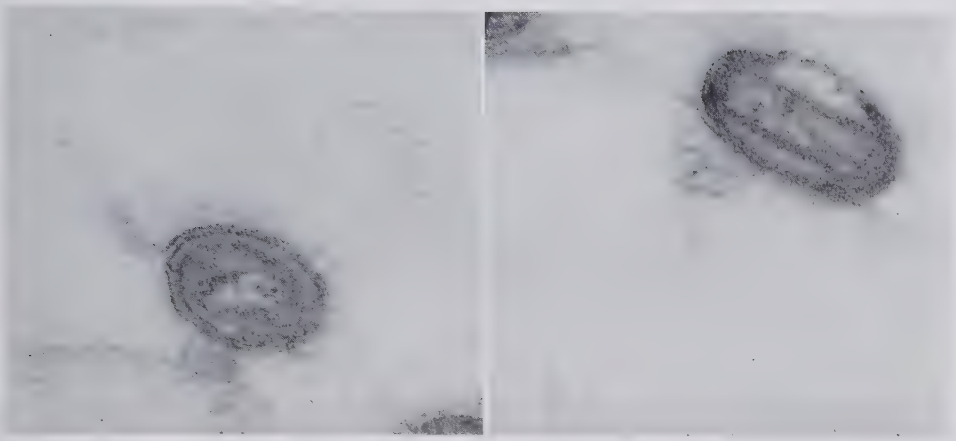


Figure 271. Vaccinia virus elementary bodies in a section of infected chorioallantoic membrane of the embryonated egg. The elementary bodies are extracellular. Note the internal structure and double limiting membrane. × 131,000. (Morgan, J. Exptl. Med.)

to bacterial recombinants, tended toward lesser virulence.[47]

Vaccinia in man. Human inoculation with vaccine lymph (see below) is usually intracutaneous by the so-called multiple pressure method in which thirty to forty punctures into the skin are made with a needle through a drop of vaccinia virus preparation about 1 cm. in diameter. After 3 or 4 days a papule appears which becomes vesicular on the sixth or seventh day, progresses to a pustule at 10 or 12 days, and then regresses. Fever may occur from the fourth to eighth day, and there may be some tenderness and enlargement of the regional lymph nodes. The crust which forms on the pustule becomes detached about 3 weeks after inoculation (foveation), leaving a red pitted scar which turns white in time. Complications are quite rare. Generalized vaccinia, with eruption at other than the site of inoculation and a more pronounced general reaction, may occur when other skin lesions, such as eczema, are present at the time of inoculation. Postvaccinal encephalitis, which is extremely rare, is similar to that following the inoculation of other immunizing preparations and is apparently quite unrelated to vaccinia.

The foregoing reaction is that given by a fully susceptible individual and is sometimes called the primary reaction. It is modified in the partially or fully immune individual to give a vaccinoid reaction in the first instance and an immune reaction in the second. The vaccinoid reaction is similar to the primary reaction but accelerated and less severe. While there are gradations in the response, in general the papule appears at 2 days, and the maximum reaction is reached between 3 and 7 days. The immune reaction consists of the development of a papule or shallow vesicle which reaches its maximum size in 8 to 72 hours, disappears without going through a pustular stage, and leaves no scar.[25] The immune reaction is given by inactivated as well as active virus and represents an allergic response to the vaccine material. It is of practical importance to emphasize that a vaccine giving the immune reaction is not necessarily active and therefore may not be an effective immunizing agent for the nonimmune.

Experimental Infections. Variola virus infects only man and monkeys but is readily cultivable on the chorioallantoic membrane of the embryonated hen's egg where it produces pock-like lesions, and it may be maintained in serial passage in the yolk sac. The disease in monkeys[60] is similar to that in man, and there is some reason to believe that natural infection may occur. Only minimal lesions are produced in the rabbit and calf. It is extremely difficult to pass the infection serially in the rabbit, a characteristic that differentiates variola sharply from

vaccinia, and on serial passage in the calf the virus loses its original character and becomes vaccinia-like. The adult mouse may be infected by intracerebral inoculation but massive doses are required to produce regular death patterns.[17] The suckling mouse appears to be more susceptible and offers possibility for the assay of infectivity and protective antibody.[84, 85]

In contrast, most laboratory animals may be infected with vaccinia virus, and it is readily cultivable on the chorioallantoic membrane of the embryonated egg where it produces pocks closely similar to those of variola. The rabbit is the usual experimental animal, and infection is produced by all the customary routes of inoculation. The virus adapts relatively readily to growth on various kinds of tissues, and coincident alterations in virulence have resulted in strains which produce encephalitis, orchitis, or pneumonia, and strains differ in other respects such as pock morphology and heat resistance.

Vaccine. Vaccine for human use is usually produced in the calf, though other animals, such as sheep, may be used also. The inoculum, or "seed" virus, is maintained at maximal activity by frequent passage in the rabbit and is used to inoculate the shaved abdomens of calves. After confluent vesicles have appeared along the scratches, the virus is harvested by scraping off the vesicle walls and contents, and this material, vaccine lymph, is emulsified in glycerol. The activity is stable for very long periods when vaccine lymph is stored at —10° C., for about 6 months at 0° C., but for little more than a week at room temperature.

The virus may also be grown for vaccine purposes on the chorioallantoic membrane of the chick embryo, or in chick embryo tissue culture.[67] Since the virus is grown under sterile conditions, problems of bacterial contamination, *e.g.*, tetanus bacilli, etc., that are inherent in calf lymph vaccine production do not arise. These culture vaccines do not seem to be as effective immunizing agents as the calf lymph preparation, possibly because of adaptive changes to chicken tissue.

The activity of vaccines is assayed by the rabbit scarification test,[74] *i.e.*, initial infection but not serial passage, but it has been urged by some that potency may be assayed equally, or possibly more, reliably by tissue culture methods.[10, 26, 75]

VARICELLA AND ZOSTER

Varicella (chickenpox) and zoster (herpes zoster, shingles) are exanthematous diseases of man characterized by a vesicular eruption and the presence of acidophilic intranuclear inclusion bodies in the affected cells. Zoster, often known as herpes zoster, is quite unrelated to herpes simplex, and both this disease and chickenpox are caused by viruses of the pox group. The characteristic brick-shape virus particles, 210 by 240 mμ, are found in the vesicular fluid.

While it has been reported that monkeys may be infected with these viruses, as yet there is no conclusive evidence that they can infect animals other than the human host. They will not grow on the embryonated hen's egg, but zoster, though not varicella, has been grown in human skin grafted onto the chorioallantoic membrane.[14, 54] The lesions produced resembled those of the naturally occurring disease, with intranuclear inclusion bodies in the affected cells, but the infection could not be transferred. It has been reported also that inclusion bodies are produced in roller tube cultures of vesicular material,[140] but serial passage required the transfer of infected cells rather than culture fluid alone, and complement-fixation tests for antigen in the culture gave ambiguous results. Since these viruses apparently do not infect experimental animals, and are at least not readily cultivable, knowledge of them remains limited.

Varicella. Chickenpox is a common, highly contagious exanthematous disease of childhood which occurs in epidemic form. After an incubation period

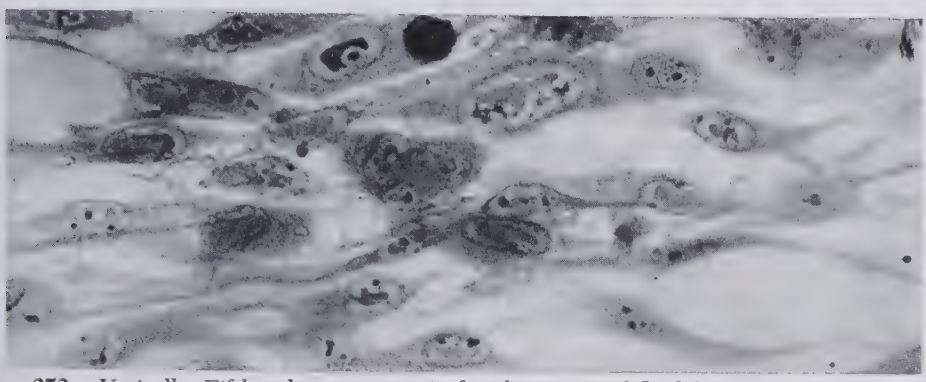


Figure 272. Varicella. Fifth culture passage in foreskin tissue of fluid from varicella lesion. Focus of cells with intranuclear inclusions on sixth day after inoculation. × 550. (Weller, Proc. Soc. Exptl. Biol. Med.)

of 2, to possibly 3, weeks, there is a febrile reaction, and a macular eruption appears which progresses to the vesicular stage and may become pustular. These lesions occur in successive crops and may be observed in all stages of development. The prickle-cell layer is affected but without the reticular degeneration which occurs in variola. As the lesion develops, the intercellular edema becomes much more marked than in the variola vesicle, an inflammatory exudate appears, and giant multinucleate cells are present. The corium may form the base of large lesions with consequent scarring, but in general the necrotic changes found in variola lesions do not occur. The lesion is unilocular even at an early stage and can be distinguished from the early multilocular vesicle of variola-vaccinia infections. In the adult there is some tendency toward a varicella pneumonia which may be less rare than commonly supposed.[76, 92]

Some neurotropic tendency of the virus is evident in this disease by the occasional occurrence of encephalitis as a complication, and there may be neuritis of the cranial nerves. The disease is not affected by chemotherapeutic drugs, but chemotherapy is useful in minimizing secondary bacterial infection of the lesions.

Zoster. The vesicular eruption of zoster, or shingles, is substantially identical with that of varicella, but the disease is distinguished from varicella by its sporadic occurrence in adults, inflammation in spinal or extramedullary ganglia accompanied by severe pain localized to some portion of the involved nerve, and the tendency of the vesicular eruption to follow the distribution of one or more cutaneous sensory nerves, usually of the torso.

The disease is initiated by a febrile reaction, general malaise, and extreme tenderness along the dorsal nerve routes. A macular eruption appears within 3 to 4 days which becomes vesicular, and pustular when secondary bacterial infection occurs. The dorsal roots of the trunk are most often affected, but involvement of the maxillary or mandibular divisions of the trigeminal nerve may occur, with vesicular lesions in the tonsillar area or buccal mucosa; the ophthalmic division may be affected, with scleritis and lesions of the cornea and conjunctiva; or involvement of the geniculate ganglion with corresponding vesicular lesions may occur. Meningoencephalitis is an uncommon complication, and persistent neuralgia tends to occur in older persons. It is clear that the neurotropic tendencies of the virus are much more marked in zoster than in varicella.

Relation of Varicella and Zoster. It has long been apparent that these diseases are intimately related. While varicella occurs largely in children and zoster in adults, contact with varicella apparently can result in zoster in adults, and, conversely, zoster seems to have been the source of varicella, at times in

epidemic form, in children. The virus particles found in vesicular fluid in the two diseases are morphologically indistinguishable, but evidence of antigenic relationship is inconclusive. Zoster may occur in children or adults who have recovered from varicella, and it is uncertain whether there is a cross-immunity between the two diseases. Some workers have reported substantially complete identity of complement-fixing antigen present in vesicular fluid, but this has not been observed by others. An immunological relation is, however, indicated by the agglutination of varicella virus particles in zoster convalescent serum, though to a lower titer than in homologous convalescent serum, and the fluorescent antibody technique has also shown the presence of common antigens in the two viruses.[141]

It may be concluded, as a working hypothesis, that (a) the two diseases are produced by closely similar viruses, one tending to be dermotropic and the other neurotropic, or (b) by the same virus, some strains of which are sufficiently neurotropic that they are able to persist in the spinal ganglia following recovery from varicella, and the latent infection may be activated by massive doses of varicella virus or other nonspecific insult. It has been suggested also that zoster represents varicella infection modified by a partial pre-existing immunity.

POX DISEASES OF LOWER ANIMALS

Pox disease occurs in a number of lower animals, including cattle, mice, and fowl, as noted earlier, and in addition in sheep, horses, swine, and a number of birds such as pigeons and canaries. In general, these diseases are characterized in the acute form by a vesicular eruption, but lesions may occur in the mouth as in stomatitis of horses, or the proliferative response to the infection may predominate as in one of the clinical types of fowlpox. In all of these diseases inclusion bodies of the Guarnieri type are found, often in large numbers, in the affected epithelial cells, and the causative viruses are morphologically closely similar and immunologically related to one another. Not all are equally well known, and only three, cowpox, mousepox, and fowlpox, will be considered here.

Cowpox. Cowpox is a mild, naturally occurring disease of cattle, characterized in the acute form by a vesicular eruption on the udder and teats. It is not a common disease, at least in the acute form. It is transmissible to man, as noted earlier in connection with Jenner's work, and the human disease is somewhat more severe than vaccinia. The virus is distinguished from variola and vaccinia in its hemorrhagic properties, apparent in the pocks formed on the chorioallantoic membrane of the chick embryo, and the vesicle fluid in bovine and human infections may contain blood. There is a cross-immunity between cowpox on the one hand and variola and vaccinia on the other, although minor antigenic differences are demonstrable. Cows may also acquire vaccinia from man.

Mousepox.[45] Mousepox, or ectromelia, is a naturally occurring infection of laboratory mice which was described as of viral etiology in 1930, and its relation to the pox diseases was demonstrated in 1945. It occurs in many parts of the world but does not seem to be present in this country as a naturally occurring infection, at least in the acute form. This disease has been particularly useful as a model experimental infection, such as for the study of the pathogenesis of pox disease, as described earlier, and as an immunizing infection comparable to smallpox or diphtheria in experimental epidemiological studies (Chap. 10).

The primary lesion develops at the portal of entry but is preceded by extensive multiplication of the virus and necrosis in the viscera, the spleen and liver particularly. In the acute form of the disease death occurs within 2 or 3 days after the appearance of the primary lesion, and on autopsy areas of necrosis

are found in the liver and spleen. If the animal does not die at this point, a rash develops, at first papular and then vesicular with ulceration, and when the primary lesion is in the footpad, as is often the case, the tissue becomes edematous, then gangrenous, and sloughs off. The mortality rate is often high, but strains of the virus vary in this respect, and the infection may persist in latent form. The large cytoplasmic inclusion bodies are numerous in affected epithelial cells.

The virus produces only a latent, inapparent infection in the rat, may be grown on the chorioallantoic membrane of the embryonated hen's egg, and rabbits and guinea pigs can be infected with egg-passage strains. It is immunologically related to variola and vaccinia and to cowpox.

Fowlpox. Fowlpox is a disease of domestic fowl which occurs in three clinical forms, *viz.*, the form in which the proliferative response results in the development of epithelioma-like growths on the comb, wattles, and skin of the head; the form in which yellowish membranous lesions are found in the mouth; and a form characterized by the presence of a watery or mucopurulent discharge from the nose and eyes known as roup.

The virus is morphologically similar to the other pox viruses, but somewhat larger than vaccinia and variola viruses. The virus particles, or elementary bodies, are known as Borrel bodies in this disease, and the large intracytoplasmic inclusion bodies are called Bollinger bodies. It is closely related to, but probably not identical with, the viruses of canary pox and pigeon pox. A variety of domestic birds may be infected experimentally, including the turkey, pigeon, goose, duck, and guinea fowl, together with a number of wild birds such as quail and pheasant. This virus, or closely related group of viruses, is set apart from the other pox viruses in that it is not pathogenic for mammals such as man, goat, sheep, mouse, rat, or guinea pig, but it is cultivable on the chorioallantoic membrane of the embryonated hen's egg.

THE HERPES VIRUSES

The group of herpes viruses includes the virus of herpes simplex affecting man, that causing a naturally occurring disease of swine called pseudorabies or "mad itch," a virus known as B virus found in monkeys which is immunologically related to both herpes and pseudorabies viruses, and virus III which occurs in rabbits. These viruses are similar in morphology, produce intranuclear inclusion bodies and a vesicular rash, and have neurotropic tendencies to a greater or lesser degree. They characteristically produce a mild disease in the natural host but highly fatal infections in other animals. It has been suggested that these viruses may be derived from a common ancestral type, differentiating by adaptation to the several host species.

HERPES SIMPLEX

Herpes simplex (herpes febrilis, herpes labialis) is a naturally occurring disease of man characterized by vesicular lesions in the epithelial layers of ectodermal tissue and the presence of intranuclear inclusion bodies in the affected cells. The virus is a single entity with possibly minor variations in antigenic character between strains that do not seem to be reflected in the epidemiology of the disease. The virus particle is a spherical or spheroidal body 100 to 150 mμ in diameter.[144] In the infected chorioallantoic membrane, the virus first appears as a much smaller particle, 30 to 40 mμ in diameter, within the nucleus. These increase in size, apparently in part by the acquisition of a mem-

brane, escape into the cytoplasm and acquire a second membrane, and finally emerge as mature particles.[95] The presence of the virus in both nucleus and cytoplasm of infected cells has also been demonstrated by the fluorescent antibody technique.[79]

In cell culture two general types of cytopathic effect are produced, (a) that characterized by the formation of syncytial giant cells, and (b) proliferative changes with the rounding and piling up of cells.[55] These appear to be characteristics of strains which may be separated from one another when they become admixed. These varieties are substantially identical with respect to antigenicity and other biological properties,[121, 122] but are reported to differ slightly in density as indicated by small differences in sedimentation rates.[107] They are known as the GC, or giant cell, and P strains.

The Disease in Man. The vesicular lesions of herpes begin as a local burning sensation, followed by the appearance of papules which become vesicular and coalesce to form groups of thin-walled vesicles. The prickle-cell layer is affected with intra- and intercellular edema, and there are capillary dilatation and infiltration of inflammatory cells in the corium but no necrotic changes. The vesicular fluid contains epithelial cells and multinucleated giant cells with inclusions present in the nuclei, and the fluid is infectious. The vesicles rupture, scabs are formed, and healing occurs without scarring.

Herpetic infection in man takes two forms regardless of the site of the vesicular lesions. The one is a primary infection, with severe, sometimes fatal, systemic effects, which occurs in individuals who do not show the presence of neutralizing serum antibody. The other, occurring in persons showing neutralizing serum antibody, is characterized by the presence of local lesions but with minor or no systemic effects.

Primary herpes. Primary infections occur relatively early in life. Infants have an effective passive immunity, and infection seldom occurs in the first 6 months of life. By 6 years of age the proportion of individuals showing the presence of neutralizing antibody is increasing rapidly, and in adults the proportion of immunes is associated with economic status, 35 to 50 per cent at high levels and 90 per cent or more in lower income groups. The incidence of the primary type of infection is not sufficient to account for the high incidence of neutralizing antibody in adults, and it is probable that the primary infection is often subclinical; Scott has estimated that as much as 80 per cent of primary infections are subclinical.

In the nonimmune the vesicular eruption commonly occurs in the mouth to give an acute herpetic gingivostomatitis (aphthous stomatitis, ulcerative stomatitis) in which the vesicular eruption ap-

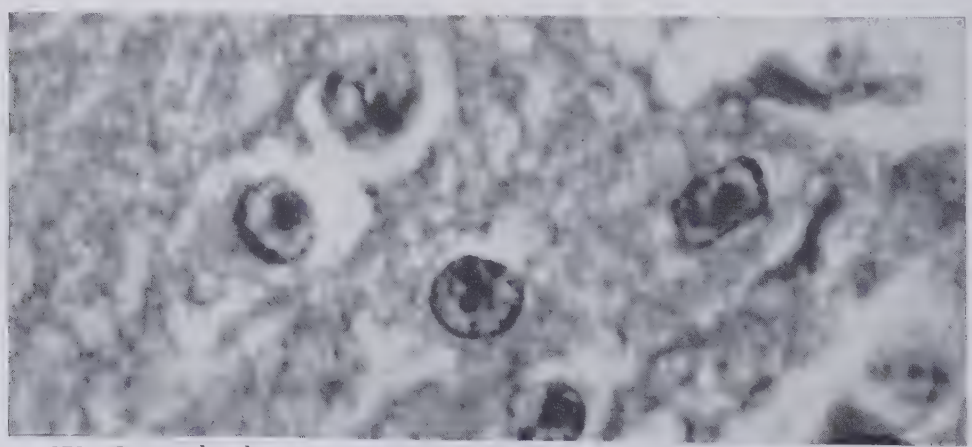


Figure 273. Intranuclear herpetic inclusion bodies in the human brain. (Armed Forces Institute of Pathology, No. 102644.)

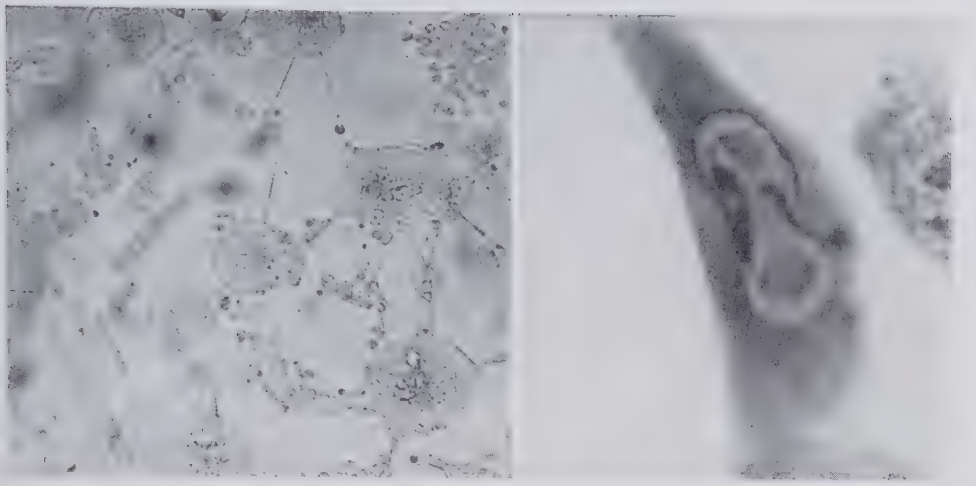


Figure 274. Herpes virus in HeLa cell culture. *Left*, one kind of cytopathology produced by the virus, characterized by the formation of multinucleated giant cells joined by cytoplasmic bridges. × 100. *Right*, an infected cell showing division of the nucleus and an inclusion body in each of the daughter nuclei. × 1000. (Gray, Arch. f. d. ges. Virusf.)

pears as plaques and shallow ulcers; the tonsillar region may be affected, and the gums are often involved. This condition is to be differentiated from herpangina of Coxsackie virus etiology (Chap. 39). The submaxillary lymphatics may become swollen and tender, and there is a constitutional reaction of varying severity, including high fever. Occasionally the virus invades the blood stream to give rise to a generalized vesicular eruption known as eczema herpeticum or Kaposi's varicelliform eruption which may simulate mild variola, generalized vaccinia, or severe varicella. The eye may be infected with the production of herpetic keratoconjunctivitis, or the central nervous system involved to give a herpetic meningoencephalitis. There is reason to believe that in the last instance the infection reaches the central nervous system via the peripheral nerves rather than by the hematogenous route. While primary herpes may be fatal, it is usually a self-limited infection, persisting for perhaps 2 weeks.

Recurrent herpes. Following primary infection, the virus persists in the tissues in spite of the presence of circulating antibody and constitutes an exception to the general rule that a solid immunity to viral infection is associated with the presence of neutralizing antibody. From the point of view of the virus, it is a highly successful parasite, persisting indefinitely in the tissues of the host.

The infection is activated from time to time in various nonspecific ways, often some febrile reaction, so that herpes may appear during convalescence from other diseases, physical shock such as chilling, metabolic or hormonal disturbances such as menstruation, etc. Under experimental conditions the infection may be activated by hypersensitivity reactions[1] or administration of adrenalin[119]; increased secretion of adrenalin occurs in many of the conditions in man associated with activation of herpetic infection. Individuals vary widely in their susceptibility to recurrent infection, *i.e.*, in the severity of the nonspecific stimulus required to induce an attack.

In recurrent herpes the disease is limited to a local vesicular lesion, presumably because of modification of the disease process by the pre-existing immunity. The eruption usually occurs at mucocutaneous junctures such as at the border of the lips or nares (fever blisters, cold sores). It may also occur on the genitalia (herpes progenitalis) of either sex, on the glans penis or corona in the male and on the labia and lower vaginal mucous membrane in the female.

Immunity. As indicated above, re-

Figure 275. Herpes virus elementary bodies in an ultrathin section of infected chorioallantoic membrane of the embryonated egg. Some particles are intact, while others show distortion produced by removal of the embedding agent (methacrylate) and the discontinuous structure of the particle. × 25,000. (Morgan, J. Exptl. Med.)

covery from primary herpes infection is associated with the appearance of neutralizing antibody in the serum which may be titrated in tissue culture.[99, 117] It modifies subsequent recurrent disease but does not prevent it. Complement-fixing antibody appears concurrently,[118] and both antibodies reach peak titer in 2 to 3 weeks. The virus, at least that grown on the chorioallantoic membrane, contains two and possibly three kinds of antigens. One of these stimulates the production of neutralizing antibody and is separable by differential centrifugation from the soluble complement-fixing antigen which also gives a skin reaction on intradermal inoculation into immune individuals. It has been reported that the soluble antigen is differentiable into a heat-stable and a heat-labile component, the latter giving the skin reaction,[65] but the evidence is not altogether consistent.[18] The utility of the skin test has not been established.

Experimental Infections. The virus may be grown on various kinds of tissue cultures, including rabbit testis tissue or kidney tissue where it forms plaques[66]; the occurrence of strains characterized by differences in cytopathic effect (see above) results in the macroplaque associated with syncytium formation, and the microplaque in which the host cells are rounded up. It may also be grown on the chorioallantoic membrane of the 12-day-old embryonated hen's egg, forming pock-like plaques on the membrane which differ from the larger, deeper necrotic pock produced by variola and vaccinia viruses; the typical lesion may not occur until the virus strain has been "egg-adapted" over two or three passages. The common laboratory animals, including the rabbit, mouse, hamster, and rat, may be infected by various routes of inoculation, the results depending somewhat upon the relative dermotropic or neurotropic tendencies of the virus strain. Inoculation of the rabbit cornea results in keratoconjunctivitis and corneal opacity, and this infection, together with that of the chorioallantoic membrane, is commonly used for diagnostic purposes. Intracerebral inoculation results in encephalitis, which may also occur in the rabbit following corneal inoculation with a neurotropic strain of the virus.

HERPETIC INFECTIONS OF LOWER ANIMALS

Among the variety of viral diseases of lower animals in which a vesicular erup-

tion occurs, at least the three noted earlier are caused by viruses closely similar to the virus of herpes simplex. Although only one of these, B virus, infects man, they may be considered briefly here.

Pseudorabies

Pseudorabies (mad itch, infectious bulbar paralysis) is a naturally occurring disease of swine which is relatively mild but highly contagious in this animal. It occurs in Europe and the Americas, and in this country appears, on the basis of neutralizing antibody surveys, to be prevalent among pigs in the Middle West. In the acute form the onset is sudden after a very short incubation period, and the disease is characterized by intense pruritus and late paralysis, with sudden death after 1 to 2 days. The disease also affects cattle, horses, goats, sheep, dogs, cats, and other domestic animals, but not man. The common experimental animals, of which the rabbit is one of the most susceptible, are readily infected by any route.

The virus is widely distributed in the body and is present in saliva, but the disease is an acute infection of the central nervous system. It progresses so rapidly that gross signs are not marked other than hyperemia of the meninges and evidence of hemorrhage there and in the lungs. The virus apparently reaches the central nervous system via the peripheral nerves, producing degeneration of the nerve and glial cells in the spinal ganglia and in the corresponding segments of the cord in rabbits. In cattle the nerve and glial cell degeneration occurs in the cerebral cortex and is marked in Ammon's horn.

B Virus

This virus was originally isolated (1934) from a fatal case of acute ascending myelitis in a laboratory worker who had been bitten by an apparently normal rhesus monkey. It was isolated subsequently directly from the monkey[89] and human infections, usually in laboratory workers, occur from time to time.[63, 101] Rabbits and guinea pigs may be infected experimentally, the virus showing strong neurotropic tendencies, and the virus may be grown on the chorioallantoic membrane of the chick embryo. The virus may be grown in various kinds of tissue cell culture; and variants, characterized by different kinds of cytopathic effects, have been described.[43] Neurotropism is less marked in the monkey, and experimental infection produces a generalized infection with a vesicular rash.

The virus is closely similar to that of herpes simplex, being of about the same size and producing intranuclear inclusion bodies in affected cells. It is immunologically related to both herpes simplex virus and pseudorabies virus. Melnick and Banker[89] found neutralizing antibody in pooled human γ-globulin and in the serum of eleven of forty persons examined in Bombay, as well as in nine of forty-four apparently normal monkeys. It is supposed that the infection is endemic in monkeys, but interpretation of serological evidence, especially in man, must take into consideration the cross-reaction with herpes simplex virus.

Virus III

This virus was found in apparently normal rabbits by Rivers and Tillett in 1923 and again by Andrewes and his co-workers.[3] The naturally-occurring infection is asymptomatic, but the presence of the virus in infected rabbits is demonstrable by serial intratesticular blind passage to eventually produce lesions in the testicles, skin, and cornea. Intracellular inclusion bodies, closely similar to those of herpes simplex, are found in the affected epithelial and endothelial cells. The virus can be grown

in rabbit testis tissue culture, producing typical intranuclear inclusions. So far as is known, it does not infect man or other animals.

FOOT-AND-MOUTH DISEASE[16]

Foot-and-mouth disease is a naturally occurring, highly contagious disease of cloven-footed animals, primarily of cattle, swine, sheep, and goats, which occasionally infects man. It is characterized by a vesicular eruption on the lips, buccal cavity, pharynx, and extremities, but the causative virus appears to be quite unrelated to the herpes viruses.

The virus is present in the vesicle fluid early in the disease and may also be present in the blood, saliva, urine, and milk, but disappears within 3 to 5 days from the local lesions and the blood. This was the first animal disease shown to be of virus etiology, by Löffler and Frosch in 1897. The virus is about 22 mμ in diameter and spherical in shape.[6] It has been grown in swine and lamb kidney, and bovine kidney and other tissue cultures, and may be assayed by the plaque method.[7] Neutralizing and complement-fixing antibody are formed by the infected animal, the former assayed by inoculation into the bovine tongue, and the antigen in the latter is infected epithelium of the tongue. The virus occurs as three main serological types, each of which has a number of subtypes.

The disease may be reproduced in the guinea pig, usually by intradermal inoculation of the footpad. A vesicular eruption occurs at the site of inoculation, constitutional symptoms appear, and a secondary eruption appears on the tongue, gums, and lips. The eruption begins to subside about 2 days after its appearance, and the lesions are healed in about 2 weeks. Infected animals are debilitated, but the disease is relatively mild in that the case fatality rate is 5 per cent or less.

The disease in cattle and other domestic animals is of considerable economic importance, not because of its fatality rate of 2 to 3 per cent, but because of its debilitating effect with consequent loss in milk and meat production. Immunization has not been a satisfactory or practical control measure, and the disease is controlled by slaughter of infected animals and rigorous quarantine, the latter including certain meat products such as frozen beef in which the virus remains infective. The virus persists in dry form and may be transported mechanically on clothing, by wild birds, etc. Human infection appears to be very rare, and the disease is relatively mild and of minor significance in man except in relation to its spread among domestic animals.

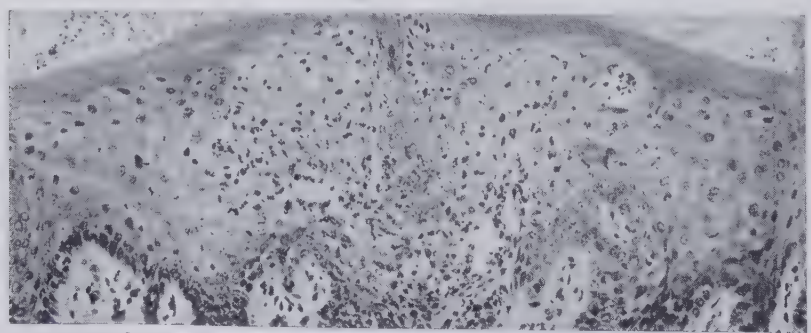


Figure 276. Foot-and-mouth disease. The section is from the tongue of a guinea pig and shows an early necrotic lesion of the epithelium. × 200. (Galloway.)

CAT-SCRATCH FEVER[103]

Cat-scratch fever (nonbacterial regional lymphadenitis, benign inoculation lymphoreticulosis, cat-bite fever) is a disease of man, apparently acquired from cats, in which there is a primary papular lesion at the site of inoculation which may progress to a small ulcer, and, in a few instances, a transient localized measles-like eruption has occurred. There is a systemic febrile reaction in about two thirds of the cases, and anorexia, malaise, weakness, and aches; occasionally the central nervous system is involved, and there is a regional lymphadenopathy. The immediate regional nodes are involved first, and subsequently secondary nodes may be affected. There is swelling and tenderness, and the nodes become indurated or suppurate and may require surgical drainage. The affected nodes are encapsulated and occur as three histological types, *viz.*, the acute caseous type or soft tuberculoid granuloma, the acute necrotizing lesion containing a core of polymorphonuclear leucocytes and no definite demarcation from the surrounding tissue, and an epithelioid lesion which is a group of epithelioid cell granulomata with germinal center hyperplasia and no evidence of cellular degeneration.

The epidemiological character of the disease suggests that it is of an infectious etiology. Most cases have a history of skin injury from a claw scratch or bite of cats, but there is no relation to an obvious illness of the animal. The apparent feline origin of the infectious agent, the nature of the disease in man, and the development of antibody fixing complement in the presence of antigens of the psittacosis-lymphogranuloma group of microorganisms, has suggested that the etiologic agent may be a member of that group. But the occurrence of such antibody is irregular, in probably not more than 50 per cent of cases, and this and the failure to react to the Frei test makes such a supposition quite uncertain.[5]

The pus obtained from infected lymph nodes, prepared similarly to the original Frei antigen, has been found to give an apparently specific skin reaction in cases of the disease.[78, 105, 138] This material agglutinates rabbit erythrocytes, and the hemagglutination is inhibited by patients' serum and by rabbit antiserum to the pus.[36] A hemagglutinating agent has been isolated and passed serially in the allantoic cavity of the embryonated hen's egg.[134] This agent has no cytopathic effect in cell cultures and produces no evidence of infection in the rabbit cornea, but its hemagglutinating activity is inhibited by antiserum to herpes simplex virus.

RUBEOLA AND RUBELLA

Measles (rubeola) and German measles (rubella) are common exanthematous diseases of childhood which are of viral etiology. The causative viruses are not related to one another or to those causing other exanthematous diseases.

RUBEOLA (MEASLES)

Measles is one of the commonest diseases of childhood, and the median number of cases reported per year in this country exceeds half a million even though many cases are probably not reported. Man appears to be universally and highly susceptible to this infection, and the disease confers a solid immunity. These two factors combine to make measles the classic example of periodicity in the prevalence of infectious disease (Chap. 10), and it tends to recur in epidemic form at 2- to 3-year intervals. The probability of acquiring the

infection is sufficiently great that the disease occurs early in life in most individuals, but some escape childhood infection, as in rural or other areas where contacts are less numerous, and the disease may appear in young adults aggregated in large groups, as in military camps.

Etiology. The viral etiology of measles was established in 1911 by Goldberger and Anderson as an agent passing Berkefeld N filters; it is a spherical particle about 140 mμ in diameter as indicated by gradacol membrane filtration. The virus had been grown in chick embryo and chick embryo tissue culture[104, 122] but without pathology. It remained poorly known because of the necessity for infectivity assay in monkeys, which are now known to be often immune, until it was isolated by Enders and his co-workers in 1954[41] in human and monkey kidney cell cultures. In such cultures it produces a characteristic cytopathic effect, the formation of syncytial masses of multinucleated giant cells containing the acidophilic intranuclear inclusion bodies. The virus grows in human amnion cells on primary isolation, and has been adapted to a variety of cell lines.[13, 50, 69] Complement-fixing antigen is found in culture fluids, and the cell culture system is used for assaying protective antibody. The virus forms a hemagglutinin for monkey erythrocytes in monkey kidney or human amnion cell cultures, but not in chick embryo fibroblasts, and hemagglutinin-inhibiting antibody occurs with other antibody activity in antiserums.[108, 109]

The virus is immunologically related to that of canine distemper which is of approximately the same size and produces a similar histopathology.[100] The antigenic relationship is such that there is cross-protection, *i.e.*, dogs are protected against distemper by immunization with measles virus, and the nature of the relationship has been of considerable interest.[53, 96, 136]

Disease in Man. The infection is probably acquired through the oropharyngeal and ocular pathways. The importance of the latter has been emphasized by Papp[9] who goes so far as to state that infection can be prevented by the ocular instillation of convalescent serum within a few hours after exposure. Replication occurs in localized areas in the lymphatic system, followed by a transient viremia during which the virus may be isolated from the blood, and the appearance of symptoms.

The disease is initiated by prodromal symptoms of a few days' duration which are essentially catarrhal in nature and include cough and fever. During this period, characteristic lesions, Koplik

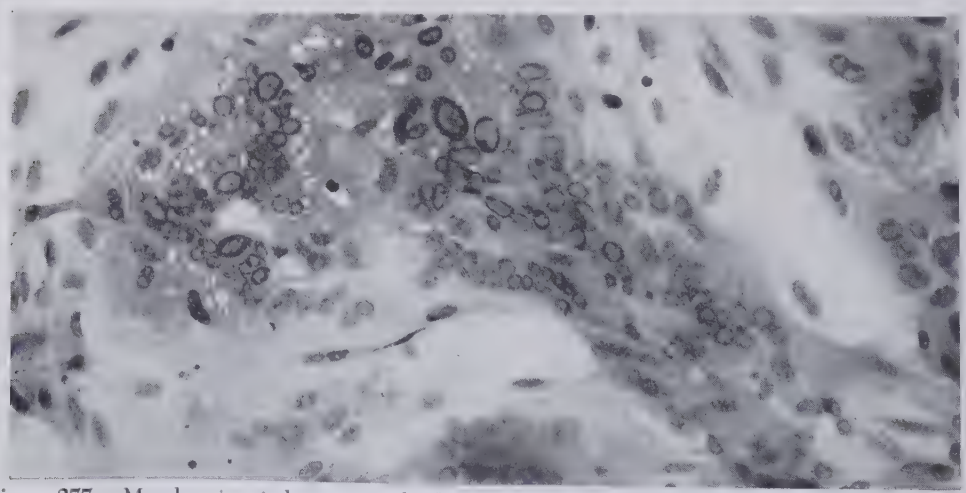


Figure 277. Measles virus in human renal cell tissue culture after 20 days' incubation showing multinucleated giant cell formation and nuclear acidophilic masses. (Enders and Peebles, Proc. Soc. Exptl. Biol. Med.)

spots, appear on the buccal mucosa. These are focal exudations of serum and epithelial cells to form vesicles which become necrotic, and their gross appearance is that of a white or bluish white center on an erythematous area. Koplik spots are diagnostic of measles and occur in 95 per cent or more of cases. Virus is present in the nasopharyngeal and tracheobronchial secretions during this period and for a day or two after the appearance of the rash.

The characteristic macular or maculopapular rash, tending to be confluent, appears within one or more days and spreads over the entire body. The capillaries of the corium are affected first, there is proliferation of epithelial cells, a serous exudate spreads into the epidermis, there is necrosis of epithelial cells, and the rash becomes vesicular. It becomes brownish in a week or 10 days, followed by desquamation.

During the course of the disease there is general lymphoid hyperplasia, and multinucleate giant cells are found in the tonsils, adenoids, lymph nodes, spleen, and appendix, which is associated with a reduced resistance to secondary bacterial infection, especially with hemolytic streptococci. Encephalomyelitis, with demyelination in the brain and cord, is an uncommon complication, with a case fatality rate of perhaps 10 per cent, but more than half the survivors are left with some impairment. Chemotherapy is useful only in minimizing secondary bacterial infection.

Immunity. Antibody is not detectable prior to the appearance of the rash, but thereafter the immune response is very rapid with the appearance of antibody within 48 hours. Recovery from the disease is associated with a solid immunity, no doubt periodically reinforced by subsequent contact with the virus, which persists essentially throughout life. Immunization by infection occurs early in life, with perhaps 20 per cent immunes among preschool children and rising rapidly to 95 per cent at 8 to 9 years of age. Complement-fixing and protective antibody, although maximal in convalescent serums, is present in almost all adult serums, and in pooled plasma or concentrated γ-globulin which may be used for passive prophylaxis. The average neutralizing and complement-fixing titer in the adult has been found to be 1:16, and susceptibles have a titer of 1:4 or less.

Immunization. If the disease is prevented, or takes an extremely light form, as a result of prophylactic administration of antibody, there is no effective active immune response. The naturally acquired disease may, however, be modified to a degree and still result in an active immunity.

The development of vaccine to give an effective active immunity has been of great interest. There are two types of vaccines, inactivated unmodified virus and living attentuated virus.[137] Interest has centered largely on the latter,[129, 130] attenuated by passage through appropriate cell systems, and it is probable that such a vaccine will become generally available.

Experimental Infections. The disease may be transmitted to monkeys, both Indian (*Macacus rhesus*) and South American (*M. cynomologous*) monkeys, by inoculation by various routes with blood or pharyngeal washings taken early in the disease, but the susceptibility of such animals is irregular. This variability is now known to be a result of the occurrence of immunizing disease in these animals, many of which are found to be immune.

RUBELLA

German measles, or rubella, is an exanthematous disease of childhood which resembles measles in many respects but is milder, *viz.*, the catarrhal symptoms of the prodromal stage are less severe, the subsequent febrile reaction is slight, and the rash is macular, discrete, or confluent and resembles that of early measles. Koplik spots are not present, and there is characteristic lymphadenitis affecting the cervical and occipital nodes, and the swelling may persist for 2 or 3 weeks.

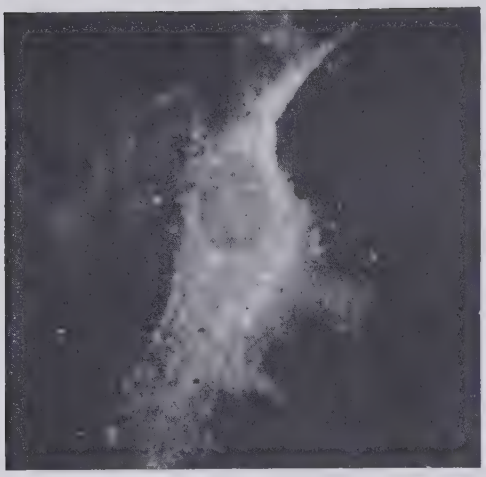


Figure 278. Intracellular measles virus in human epithelial cell tissue culture, treated with convalescent serum and with fluorescein-labeled antiglobulin serum. × 480. (Rapp and Gordon.)

The disease is regarded as of viral etiology since it has been produced in human volunteers and in monkeys by the inoculation of bacteria-free filtrates of nasal washings or blood. Attempts to grow the virus on the chorioallantoic membrane of the embryonated egg have given contradictory results, and in any case lesions do not appear to be produced to give a reproducible cytopathic effect.

For many years after its differentiation from scarlet fever and measles, rubella was regarded as an extremely mild disease of minor importance. It was observed by Gregg in Australia in 1941, however, that the occurrence of the disease in women during the early months of pregnancy is associated with a high incidence of congenital malformations, including cataract, microcephaly, deafness, cardiac and other defects of the circulatory system, as well as with abortion and stillbirth.[15, 64, 102, 106, 123]

According to the Australian figures, the proportion of malformations ranges from 83 per cent in the first month of pregnancy to 61 per cent in the fourth month. In this country similar studies have indicated a rate of about 50 per cent when the mother is infected in the first trimester, but this incidence does not reflect defects occurring, or apparent, later, such as deafness, which enter into the rates observed in Australia. In any case, it has become quite clear that rubella contracted in the early months of pregnancy frequently has serious consequences to the child. There is some evidence that γ-globulin will prevent rubella when given not later than the eighth day of the 2- to 3-week incubation period, but this is less firmly established than in the case of measles.[77]

These effects are presumably due to intra-uterine infection affecting the differentiation of embryonic tissues and structures, most often affecting the ectodermal and mesodermal layers. Autopsy findings on affected infants have included defects of the circulatory system such as patent foramen ovale and ductus arteriosus, and in one case with deafness the primitive cells failed to differentiate to form the organ of Corti.

INFECTIOUS MONONUCLEOSIS[80]

Infectious mononucleosis (glandular fever, monocytic angina) is an acute, presumably infectious, disease occurring in children and young adults characterized by an increased number of monocytes and large lymphocytes in the blood, angina, lymphadenitis, and the presence of heterophile antibody in the serum. It is known in Japan by a variety of names, including Tokushima fever and Kagami fever. A similar disease of lower animals in which the central nervous system may be involved with some frequency is caused by the bacterium *Listeria monocytogenes* (Chap. 27) which also occurs as a natural infection of man. This microorganism is not found in the great majority of cases of human disease with the syndrome characterizing infectious mononucleosis. While

there is no definitive supporting evidence, it is generally assumed as a working hypothesis that this disease is of viral etiology.

The Disease in Man.[19, 23] The onset of the disease may be acute or insidious and is characterized by loss of appetite and fatigue. Systemic involvement is indicated by the febrile reaction and symptoms such as epistaxis, nausea and vomiting, and headache; the cervical lymph nodes become swollen and tender, and there is angina ranging in severity from mild hyperemia to a pseudomembranous ulcerating form. A cutaneous rash occurs in some individuals, and there may be conjunctivitis and/or evidence of liver damage, and the central nervous system is occasionally involved. The white blood cell count increases, to as much as 80,000, the differential count showing a decrease of polymorphonuclear leucocytes and a marked increase in monocytes to give a characteristic blood picture.

The serological evidence of infection is an increased titer of heterophile antibody, demonstrable by agglutination of sheep erythrocytes (the Paul-Bunnell test) over the normal titer of 1:50 to 1:60; a titer of 1:80 is generally regarded as the minimum which may be taken as diagnostic. The hemagglutination test may be made more specific by a preliminary absorption of the serum with boiled guinea pig kidney tissue which removes "nonspecific" antibody; the antibody associated with this disease is absorbed by treatment of the serum with boiled sheep erythrocytes. The goat erythrocyte is reported[135] to give a more sensitive test, and a number of variations, including an ox erythrocyte hemolysis test[93] and the use of trypsinized bovine red cells[133] have been suggested.

The disease is thought to be transmitted by intimate contact such as kissing, and it has in fact been called "kissing disease." It is not ordinarily possible to trace contact between infected persons; this has been interpreted by some to be indicative of a low degree of contagiousness and by others to suggest that subclinical infection is very common.

Experimental Infections. Transmission of the infection, evidenced by mild lymphadenitis and small increases in the monocyte count, to the monkey has been reported by some workers but not by others. It has not been possible to cultivate an etiological agent in the embryonated egg, and human volunteer experiments have given equivocal results. Thus there is little support for the assumed viral etiology of the disease.

SALIVARY GLAND DISEASE VIRUSES[125]

A disease known as salivary gland disease occurs widely in man and in a number of animal species examined, including rats, mice, guinea pigs, hamsters, Cebus monkeys, and chimpanzees. It is characterized by cellular gigantism and intranuclear inclusion bodies (and sometimes small compact intracytoplasmic inclusion bodies) present within the affected cells, whose size and structure tend to set them off from the inclusion bodies observed in other virus diseases.[20, 82] This disease, also known as cytomegalic inclusion disease, appears to be caused by a group of closely related, but highly species-specific, viruses.

The naturally occurring infection in lower animals seems to be almost invariably asymptomatic. It is probably usually subclinical in man also, but severe disease occurs in infants,[58] commonly in those under 2 years of age, and about 100 fatal cases have been reported in all; in older children it may be secondary to some other disease.[126] The infection is generalized, and the typical histopathology is found at autopsy in the salivary, most often submaxillary, glands,

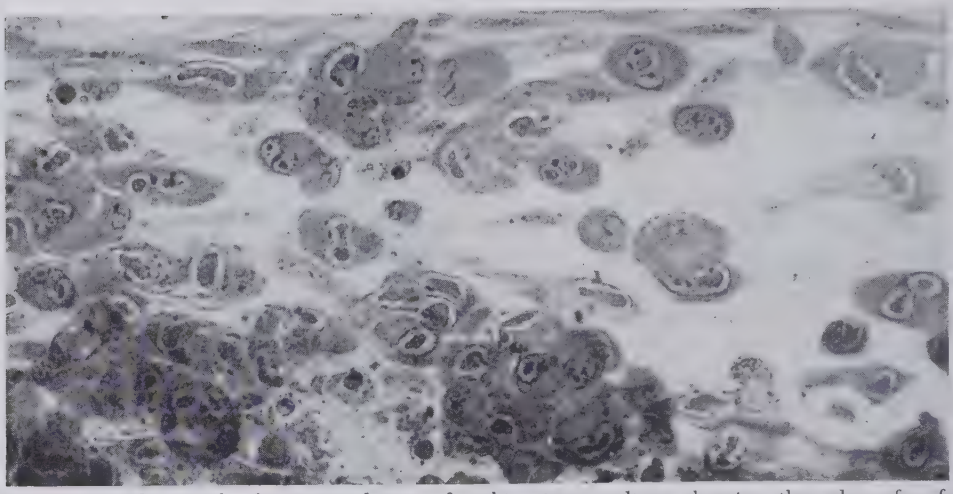


Figure 279. Salivary gland virus in human foreskin tissue culture showing the edge of a focal lesion, with central degeneration and infected cells at the periphery. Hematoxylin and eosin; × 360. (Weller, Proc. Soc. Exptl. Biol. Med.)

in the kidneys, liver, lungs, pancreas, and occasionally in the brain. The virus is found in the saliva and urine.[73, 112]

The causative viruses have been isolated in salivary gland disease of the mouse, guinea pig, and man, in the last from salivary glands, kidneys, and adenoids, in tissue culture of homologous fibroblasts of varied tissue origin.[111, 124] After not less than 30, and up to 70, days of incubation, focal areas of degeneration appear on the fibroblast sheet which are separated by normal cells and which may become confluent on continued incubation. The cells in the affected areas contain the typical large acidophilic intranuclear inclusion bodies. While such cultures may be maintained in serial transplant, the virus grows only on homologous fibroblasts, *i.e.*, mouse virus will not grow on human fibroblasts, or human virus on mouse fibroblasts, and cannot be grown on the usual HeLa cell, monkey kidney, or rabbit trachea tissue cultures. Active virus is present in the homologous fibroblast cultures; intraperitoneal inoculation of young mice with cultured mouse virus produces a fatal disease characterized by visceral necrosis; and a fatal infection is produced by the intracerebral inoculation of fetal or very young guinea pigs with homologous culture virus, but human virus will not infect these animals.

The cytopathogenic effect in fibroblast culture is inhibited in the presence of antiserum, and antigen fixing complement in the presence of antiserum is present in tissue cultures. In contrast to some other viruses, there is a close correlation between complement-fixing and protective antibody. By such tests the human strains of this virus are shown to be immunologically distinct from varicella and measles viruses and show a degree of serological heterogeneity among themselves.[142]

The general occurrence of the disease in man is indicated by the incidence of typical histopathology in 10 to 30 per cent of infants at autopsy regardless of the immediate cause of death, the isolation of the virus from apparently normal adenoidal tissue, and the distribution of complement-fixing antibody. The observed incidence of significant titers of this antibody has been 14 per cent in the 6- to 23-months age group, 53 per cent in the 18- to 25-year age group, and 81 per cent in persons over 35.

TUMOR VIRUSES[2, 31, 33, 57, 87, 110, 131]

The property of some viruses of inducing proliferation of the affected cells has been alluded to earlier.[28] In many instances proliferation is followed by necrosis, relatively rapidly in a disease such as smallpox in which the proliferative stage is so transitory as to be almost inapparent, and more slowly in the epitheliomatous type of fowlpox infection. In infections with other viruses morphologically closely similar to the pox viruses, molluscum contagiosum in man and myxoma and fibroma in rabbits, the proliferative response predominates, but necrosis occurs eventually.

Still other viruses, notably that of rabbit papillomatosis,[132] enter into a continuing, benign relationship with the host cell, and an orderly proliferation may continue for extended periods, but eventually the growth becomes disorderly, invades contiguous tissues, metastases occur, and the rapidly proliferating cells develop as a malignant process, *i.e.*, the proliferative process has become cancerous. The mammary carcinoma of the mouse associated with the virus-like milk factor[30] is similar in that the activity present in the milk produces no immediately apparent effect but is essential to the subsequent development of the carcinoma. In still other instances, such as avian sarcoma[61] and leucosis,[12] the proliferative response is malignant substantially from the initiation of the viral infection. Thus a continuous series of types of cellular proliferative response to viral infection may be constructed.

The relation of the virus to unrestricted proliferation is not clear, for evidence of its indefinite persistence in the affected tissues may be equivocal or lacking. In some instances the virus may not be directly separable from the affected tissues, but its continued presence and increase in amount are inferred from rising antibody titer to it in the blood, the infection persisting in masked form presumably because of the concurrent presence of antibody. In the case of avian leucosis virus prepared and studied in highly purified form by Beard, Sharp, and their colleagues, the viral agent, although extrinsic to the host cell and not arising *de novo* from it, nevertheless contains host tissue antigen as an integral portion of the viral substance, and the differentiation of virus and host cell tends to blur. In still other instances, the virus is detectable only in the earlier stages of the neoplastic process, and thereafter there is no evidence of its presence; under such circumstances the virus would appear to function, in current terminology, as a carcinogen.

The viruses producing disease in which the proliferative response is the outstanding feature appear to be sharply species-specific in that the infection is, with certain exceptions, not reproduced experimentally in heterologous species, and some show strongly restrictive tissue specificities within the host. Of those known at the present time, only molluscum contagiosum occurs in man, and the validity of any analogies between virus-induced neoplastic disease in lower animals and similar disease in man is dubious. Of these virus diseases, only representative forms will be described here.

MOLLUSCUM CONTAGIOSUM

Molluscum contagiosum is an infectious disease of man, world-wide in distribution and occurring in both sporadic and epidemic form. The infection is spread by contact, and the disease is characterized by the occurrence of multiple nodules on the face, arms, back, and buttocks which are at first red, then pearly white, and undergo necrosis with the discharge of caseous material. The disease is chronic, and the lesions persist over several months.

The lesion develops in the epidermis as thickened areas of hyperplasia and hypertrophy of the affected cells. There is little or no reaction in the corium in the absence of secondary bacterial infection,

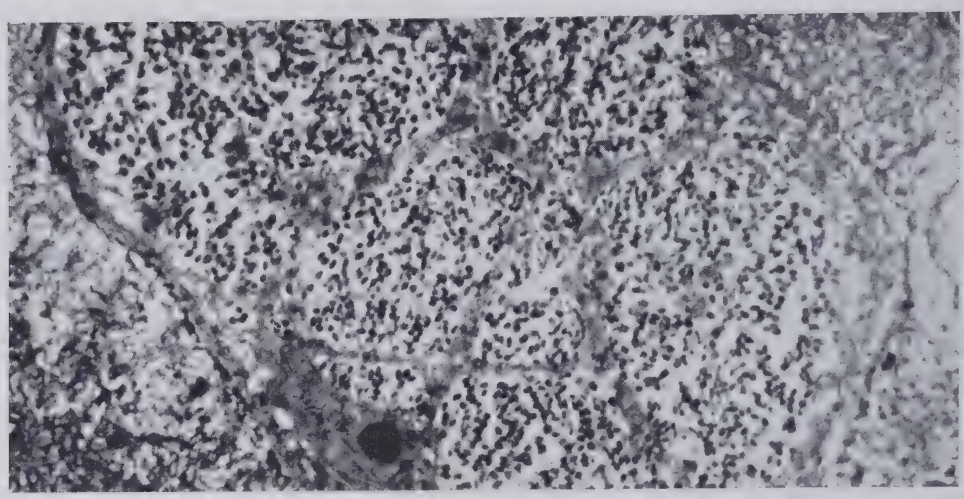


Figure 280. Electron micrograph of cell infected with molluscum contagiosum virus. Strands of cytoplasmic material separate the pockets of mature virus, and the nucleus is pushed to the edge of the cell. × 8500. (Gaylord and Melnick, J. Exptl. Med.)

and the pathological changes become more marked proceeding from the germinal layer to the surface of the epidermis. The affected cells are enlarged and practically filled with an acidophilic granular inclusion body, or molluscum body, similar to those observed in the pox diseases.[8, 22] The inclusion body contains oval or brick-shaped elementary bodies 230 by 330 mμ in size. In ultrathin sections the elementary bodies are heterogeneous with respect to contained electron-dense material, and the several morphological types may be interpreted as representing a sequence of developmental stages as described elsewhere[51, 88] (Chap. 4).

It has not been possible to infect experimental animals with this virus nor to grow it on the chorioallantoic membrane of the embryonated egg. Heat-labile soluble antigen may be prepared as extracts of macerated infected tissue which fixes complement in the presence of serum of some patients and has been shown to be unrelated to the antigens of the pox viruses.

MYXOMA AND FIBROMA VIRUSES

Infectious myxomatosis of rabbits is a naturally-occurring mild disease of the South American rabbit (*Sylvilagus braziliensis*) and a rapidly fatal disease of the European wild rabbit (*Oryctolagus cuniculus*) and the common laboratory rabbit derived from it. The disease was described by Sanarelli in 1898 as occurring in laboratory rabbits in Montevideo. Infectious fibromatosis is a naturally-occurring benign disease of wild cottontail (Sylvilagus species) rabbits, described by Shope in 1932, characterized by fibromata which eventually regress. The viruses causing these diseases are closely related immunologically, are morphologically indistinguishable, are transmitted by arthropods, and the fibroma virus may be transformed to myxoma virus.

Infectious Myxomatosis. While the virus of myxoma produces only benign tumor-like masses in Sylvilagus rabbits, the infection in Oryctolagus rabbits produces a generalized disease that is almost uniformly fatal in 1 or 2 weeks. The naturally-occurring infection is transmitted mechanically by biting arthropods, including mosquitoes and rabbit fleas, and artificial infection may be produced by pricking the skin with a contaminated needle.

After multiplication at the site of inoculation, the virus spreads through the lymphatics and into the blood stream and is distributed throughout the body.

Both epithelial and connective tissues are affected, with destruction of epithelial cells and proliferation of connective tissue. The lesions in the skin, both primary at the site of inoculation and secondary in the skin, especially about the face and anogenital region, in the lymph nodes, and in the spleen, are tumorous growths. These are characterized by varying degrees of inflammation, the deposition of a glairy mucinous fluid that appears in stained sections as a fibrinous exudate, and the presence of characteristic stellate cells sometimes called myxoma cells.

The myxoma cells are enlarged macrophage-like cells with a heavily staining nucleus, and they contain the virus, sometimes as dispersed elementary bodies in the cytoplasm, but more commonly in intracytoplasmic inclusion bodies closely similar to those found in the pox diseases.[49] The elementary bodies are oval or brick-shaped particles, of the same order of size as those of the pox diseases and molluscum contagiosum.

The virus may be grown on the chorioallantoic membrane of the embryonated hen's egg where it produces pock-like plaques, and it grows in rabbit kidney tissue culture,[21] producing inclusion bodies in typical stellate cells. A heat-labile soluble complement-fixing and precipitating antigen is formed in culture and is present in infected tissues and also in the serum of infected animals, but antibody to this antigen is not identical with protective antibody. The virus is closely related immunologically to the fibroma virus (see below).

Myxomatosis has been of considerable epidemiological interest following its deliberate introduction, in 1950 and 1951, into Australia for biological control of the native rabbit population. The unique opportunity to study the natural history of an infectious disease has been thoroughly exploited by the Australian workers.[48] The disease was also introduced in France in 1952 and spread rapidly throughout Europe.

Infectious Fibromatosis. This disease, which is transmissible to laboratory (Oryctolagus) rabbits, is similar to infectious myxomatosis, but it is not fatal. The characteristic lesion is a benign subcutaneous spindle-cell fibroma in which there is infiltration of inflammatory cells and hyperplastic and degenerative changes in the overlying epidermis reminiscent of the lesions of molluscum contagiosum in man. Closely similar intracytoplasmic inclusion bodies occur in the affected cells, and also in infected cells in rabbit testis tissue culture, but the virus does not grow in cultures of human foreskin or monkey kidney tissues.[71] The contained elementary bodies are closely similar to those of the myxoma and pox viruses.

The fibroma virus is immunologically closely related to, but not identical with, the myxoma virus. Animals recovered from fibromatosis are partially immune to myxoma in that they do not contract the disease, but they may be infected and become healthy carriers of myxoma virus. The few rabbits which recover from myxomatosis, however, appear to be solidly immune to fibromatosis.

Fibroma-Myxoma Transduction.[72] The intimate relation between the viruses of fibroma and of myxoma is indicated by the transformation of the former to the latter by a process which is apparently analogous to the *in vivo* transduction of pneumococcus types, etc. (Chap. 7). This was first shown in the rabbit by the simultaneous inoculation of heat-inactivated myxoma virus and living fibroma virus in order to give myxomatosis in the rabbit. This transduction has also been carried out in rabbit testis tissue culture. The transforming agent prepared from myxoma virus is sedimentable by high speed centrifugation and associated with a particle similar in size to that of myxoma virus. It has been suggested that the transforming principle, which is resistant to heat and ether treatment, consists of intact DNA contained within a membrane of denatured protein.

The result is necessarily an alteration in the directed synthesis of viral substance by the host cell initiated by the presence of the inactive virus, and it is

of special interest that this can occur in the more complex system involving the host-parasite relationship as well as within the self-contained synthetic mechanisms of bacteria.

RABBIT PAPILLOMA VIRUS[132]

Infectious rabbit papillomatosis, described by Shope and Hurst in 1933, is transmissible by cell-free filtrates and of viral etiology. The virus has been prepared and studied in highly purified form[33] and found to be a spherical particle, 40 to 50 mμ in diameter, morphologically indistinguishable from the viruses found in human warts.[90, 91] It resembles other viruses in chemical composition, containing 8.7 per cent nucleic acid of the deoxypentose type, but less lipid, 1.5 per cent, than many other viruses.

Infectious Papillomatosis. The disease in cottontail rabbits is characterized by warty growths on various parts of the body. It may be transmitted serially in these animals but not all are susceptible, and in half or more of those infected there is eventual regression of the papillomata, and malignant change is rare. The domestic rabbit is much more susceptible, for papillomatous disease develops readily, the tumors grow in benign form for some months and then become malignant, killing the animals. The disease in the domestic rabbit usually cannot be transmitted by cell-free filtrates in serial passage, and the presence of the virus in the affected tissue is indicated by the antibody response.

Pathogenesis of papillomatosis. The pathogenesis of this disease has been one of the most thoroughly studied of diseases of this kind, and in at least some respects may be regarded as a prototype. The histopathology has been summarized by Kidd.[70] On inoculation of the scarified skin the virus induces a prompt and marked proliferation of the epidermal cells, those of the stratum spinosum showing perinuclear vacuolation and dyskeratosis, but the cells mature to a point. They do not form the characteristic horny layer, but rather an atypical cornification occurs in which each cell is surrounded by a layer of keratin, fuses with adjacent cells to form a honeycomb structure, and the cell contents

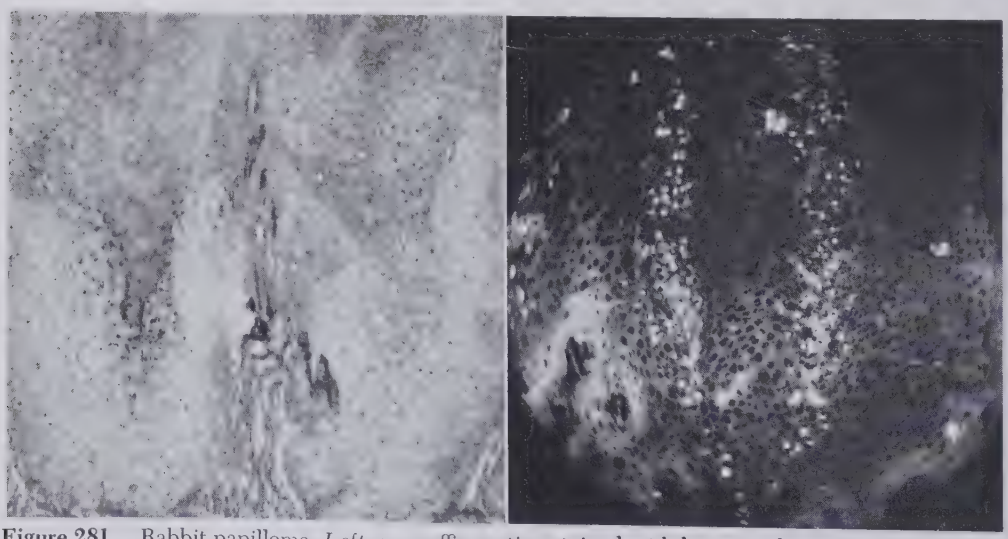


Figure 281. Rabbit papilloma. *Left,* a paraffin section stained with hematoxylin and eosin and showing the basal, granular intermediate, and superficial keratinized epithelial layers of the papilloma. *Right,* a frozen section treated with fluorescent antibody for the Shope viral antigens; the localization of the antibody in the nuclei of the keratinized epithelial cells of the papilloma is shown by the bright areas. × 100. (Mellors.)

disappear. With extension at the base, the developing cells are pushed into folds and papillae, and keratinization without desquamation results in the horny mass rising above the level of the skin and constituting the benign papilloma. Viral antigen is demonstrable in the nuclei of the keratinized epithelial cells by the fluorescent antibody technique as illustrated in the accompanying figure (Mellors).

The benign papillomata develop and persist in this form for some months, but eventually there is an abrupt change in the growth, occurring within a relatively short time in all of the lesions on a given animal. Histologically, the germinal cells, which to this point are arranged in an orderly syncytium, assume a disorderly arrangement, are devoid of perinuclear vacuoles and keratohyalin granules, and do not undergo the abnormal cornification described above. They displace the cells of the basal and spinous layers and undermine the horny mass which sloughs off. This new type of cell then breaks through the basement membrane, invading contiguous tissues, and metastasizes and assumes the character of a squamous cell carcinoma. Until this last stage, serological evidence indicates that the virus persists in the affected tissue, both in the orginal animal and in recipients of tissue transplants, but eventually the virus seems to disappear entirely.

Factors affecting malignancy. While it seems clear that at least the initial proliferative response which gives rise to the benign tumor is induced by the virus, there is no evidence that the virus plays a significant part in the subsequent change to malignancy, and there is some reason to believe that other factors may be involved. For example, the benign papilloma can be made rapidly cancerous by the application of carcinogens such as methylcholanthrene and tar, and, conversely, the virus can infect tar-induced epithelial tumors to bring about an abrupt change to malignancy; in the latter instance the virus would appear to behave as a carcinogen.

VIRAL AGENTS OF MOUSE NEOPLASTIC DISEASE

The mouse suffers from a variety of neoplastic diseases, susceptibility to which often has a genetic background, and has been an extremely useful experimental animal.

Polyoma.[40, 127] Various kinds of tumors may be produced in mice by the inoculation of cell-free extracts or filtrates of mouse tumors, including leukemic tissue, shortly after birth. For the most part multiple effects are produced, *i.e.*, it is ordinarily not possible to predict the location or character of the neoplastic growth so produced.

A possible viral nature of the activity present in such cell-free preparations is suggested by its small size and separation from intact tumor cells. The activity demonstrable in cell-free preparations of several mouse tumors has been found to be cultivable in mouse embryo tissue culture,[40] producing a cytopathology characterized by the appearance of small patches of pyknotic cells which progresses until the majority of the cells are affected in 2 weeks. The activity increases from 10^3 TID_{50} at 2 weeks to as much as 10^6 TID_{50} at 3 weeks, and the infectivity for intact experimental animals is correlated with the cytopathogenic effects and is neutralized by antiserum. The viral agents so cultivated are nonspecific with respect to the type of neoplastic growth produced in the intact animal and have been designated polyoma virus. The virus is spherical, 30 to 40 $m\mu$ in diameter, and resembles other virus in its properties.

A total of twenty-three kinds of different primary neoplastic lesions, including pleomorphic tumors of mucous glands, adenosarcomas, renal and bone sarcomas, epidermoid carcinomas, etc., are produced. It is of interest also, and in contrast with the genetic element in the occurrence of mammary adenosarcoma in the mouse, that such growths are produced regardless of strain of mouse and can be produced also in hamsters.

Animals can be passively immunized to the virus providing that antiserum is given prior to, or admixed with, the inoculum, but not when serum is given as early as one hour after inoculation. The infection appears to be common in mice in latent form, perhaps because of immunity, initially of maternal origin. Virus activity is present in the neoplasm, but when the tumor is passaged by transplantation viral activity may disappear in the course of time, perhaps ten transplants, even though the tumor remains transplantable. The nature of refractoriness to transplantable tumor is not dependent upon antibody and is of particular interest.[59]

Leukemia.[32, 94] Although Rous described cell-free transmission of chicken sarcoma many years ago, interest in this aspect of neoplastic disease remained essentially dormant until Gross demonstrated the viral etiology of a mouse leukemia in 1951 to initiate a period of intense interest and activity. Since then a number of leukemogenic viruses, active in various inbred strains of mice, have been described. The majority produce lymphocytic neoplastic disease, but some produce reticular cell neoplasms and myeloid neoplasms. These strains have been derived from spontaneous leukemias and other neoplastic disease, and from such disease occurring in irradiated animals. They differ somewhat in particle size, over a range of 70 to 100 mμ, and are immunologically distinct but show some interrelationships. They are commonly identified by the name of the investigator(s) who originally described them; the first is the Gross strain, and others as the Kaplan, Schoolman and Schwartz, Moloney, Friend, etc., strains. The origins and properties of these strains are summarized in the accompanying table.

Murine Leukemia Viruses*

STRAIN (INVESTIGATOR)	ORIGIN	HOST SUSCEPTIBILITY			PARTICLE SIZE	NEOPLASTIC CELL TYPE
		Age		Strain		
		Suckling %	Adult %		mμ	
Gross						
Passage A	AK, C_58 (spontaneous leukemia)	62–100	44–60	C_3H, C_3Hf (Bittner) (rats)	89	lymphoid
Passage X	C_3H/Gs (x-irradiated)	65		C_3H		lymphoid
Schoolman and Schwartz	Swiss, C_3HeB (x-irradiated)		15–100	Swiss, C_3H	102	lymphoid sarcoma
Kaplan	$C_{57}BL$/Ka (x-irradiated)	69		$C_{57}BL$/Ka, F_1 hybrids		lymphoid
Breyere and Moloney	plasma cell neoplasm (70429)	100	100	BALB/c, C_3H, (rats)	100	lymphoid
Moloney	sarcoma 37	100	100	BALB/c, C_3H, C_3Hf, I, C_{57}-BL, DBA/2, RIII Swiss	98	lymphoid
Friend	Ehrlich sarcoma		85–100	Swiss, DBA/2	90	reticulum—erythroblastosis
Stansly	Ehrlich sarcoma	92–100		BALB/c		reticulum cell sarcoma B
Graffi	Sarcoma I, Sarcoma II, SOV 16, Ehrlich carcinoma, Sarcoma 37	70	70	Agnes Bluhm, sg, db, (rats)	70	myeloid

* Modified from Moloney.[94]

Mammary Carcinoma.[29, 30] Three factors are involved in the development of mammary adenocarcinoma of the mouse, *viz.*, the genetic constitution of the animal, estrogenic stimulus, and a filterable activity present in cell-free preparations or milk factor. While the milk factor persists and increases in amount in the tissues of the host, hyperplasia of mammary acini occurs only in conjunction with estrogenic stimulation. Some of these become malignant adenocarcinomas, but the relation of the milk factor to malignancy is not clear.

Attempts to characterize the activity have been only partially successful. It is widely distributed in the tissues of mice harboring it, and various tissue extracts are as effective as milk. It obviously increases in amount in that it is transmitted serially in mice under natural conditions. It passes the usual bacteria-proof filters such as the Seitz and Berkefeld. It is highly active, in dilutions as high as 10^{-6} in breast tissue extracts. The activity is resistant to tryptic digestion and is destroyed by heating tissue extracts to 60° C. for 1 hour. It is sedimented at 23,000 $\times$ G in 90 minutes, and some workers have reported that the activity is associated with spherical particles about 30 mμ in diameter, but there is no unequivocal evidence that these represent virus particles. Cell-free activity does not replicate in the embryonated egg but may be recovered from tumor tissue carried for as many as eleven passages in the chick embryo. Attempts to characterize the activity chemically and immunologically have given essentially negative results.

VIRUSES OF AVIAN NEOPLASTIC DISEASE[11, 114]

The viral etiology of what is now known as the avian leucosis complex was described by Ellermann and Bang in 1908 and the fowl sarcomas transmissible by cell-free filtrates by Rous in 1911. The former is an infectious disease characterized by an autonomous proliferation of cells of hematopoietic tissues. The Rous sarcomas have been thought to originate in monocytes or histiocytes, but there is evidence[115] that the host cell of the causative virus is the fibroblast. In contrast with rabbit papillomatosis, malignancy is apparent at the initiation of these diseases.

Avian Leucosis.[12] Avian leucosis is not a single disease, but a complex of a variety of forms of disease. Beard, Sharp, and Eckert[33] divide this complex into two categories, the one lymphomatosis and the other erythromyeloblastic leucosis. In the former, cells of lymphoid origin are involved, the affected cells occur in fixed tissues rather than in the circulation, and the disease process varies with respect to the localization of the primitive cells affected, *viz.*, neurolymphomatosis, visceral lymphomatosis, isolated lymphosarcomatous tumors, etc. The second group is made up of erythroblastosis and myeloblastosis, or a combination of both as erythromyeloblastosis, and is characterized by the occurrence of very large numbers of primitive cells in the circulating blood.

While these diseases "breed true" in that cell-free filtrates of lymphomatosis produce lymphomatosis, and erythromyeloblastosis is similarly reproduced, cellular transfer of the former has resulted in the latter disease. It is quite uncertain whether the several differentiable conditions are produced by a single virus, by variants of a single virus, or by a group of closely related viruses.

Erythromyeloblastosis. While a particulate component has been isolated in studies on lymphomatosis, the most detailed studies have been carried out with the virus of erythromyeloblastosis, and it may be considered here as representative.

There is a great technical advantage in the study of this virus for it is present in the plasma of infected chickens in such large amounts that the plasma is literally turbid, and it may be sedimented by high-speed centrifugation. The virus particle is spheroid in shape, with an average diameter of about 120 mμ, and is apparently a relatively frag-

ile structure for it is pleomorphic in dried preparations like the virus of Newcastle disease.[34, 35] Its chemical constitution is not known with precision, other than its relatively high content of lipid, as much as 30 per cent, in contrast to rabbit papilloma virus. It is of special interest that it contains adenosinetriphosphatase activity which appears to be an integral part of the virus particle.

The serum of infected chickens does not ordinarily contain antibody to the virus in regularly demonstrable amounts, but it functions as an antigen in concentrated preparations. Three kinds of antigens are found, one also present in normal chicken tissue, heterophile or Forssman antigen, and virus-specific antigen. The occurrence of host-specific antigen in purified preparations of viruses is commonly assumed to be, and probably often represents, contamination. Such antigen present in influenza virus is suspected to be an integral part of the virus particle, but in the case of this leucosis virus antibody to the chick tissue antigen and that to Forssman antigen, as well as antibody to the virus-specific antigen, neutralize the virus activity which appears to establish all three kinds of antigens as structural components of the virus.

Fowl Sarcoma.[61, 113] Rous and his associates were able to transmit several types of chicken sarcomata by means of cell-free filtrates, to produce in the recipients under appropriate experimental conditions, the same kind of tumor. That designated tumor 1, which has been used extensively for experimental purposes, was a spindle-cell sarcoma characterized by free metastasis and the production of disease fatal in about a month. Tumor 7 was an osteochondrosarcoma, and tumor 18 a spindle-cell sarcoma characterized by the presence of blood sinuses in which growth occurred.

It was early observed that, after several transplants, variations began to occur in tumor 1 as indicated by the appearance of spherical rather than spindle cells and hemorrhagic growths, changes possibly attributable to variations in host resistance as well as in the tumor. Other variations may be produced experimentally. When inoculated in very large amounts in ducks this virus produces two kinds of tumors. The one appearing only after several months is no longer effective in adult chickens and produces a generalized disease characterized by periosteal sarcomata in the duck, while the other which appears much earlier is not found to be modified on subsequent infection of the chicken. The variation apparent in the first instance can be reversed by inoculation of the variant into young chicks to produce again the two types of lesions found in ducks, one of which represents the reversion. Other variants produced in ducks have been found to have altered tissue specificities in chickens, and still other variants have been produced in turkeys and guinea fowl.

The virus of tumor 1 has been prepared in purified form by both chemical and physical methods, beginning with its salting out by half-saturated ammonium sulfate in association with a nucleoprotein fraction. There appears to be some variation in the size of the infective particle, and it is usually given as within the range 70 to 100 mμ. Particles of this size have been reported to occur in the affected tissue, for example as round or oval bodies within the walls of vacuoles of the cells, showing a double limiting membrane surrounding a central electron-dense body,[42] but in general it has not been established that such bodies are virus particles. Purified preparations of the virus give about 8 per cent total nitrogen, 35 per cent lipid, and 1 to 2 per cent pentose nucleic acid. The virus has been grown in tissue culture,[83] high dilutions of inoculum producing, in chick embryo tissue culture, discrete foci of cytopathology in the monolayer, characterized by a swelling and granularity of the cells and eventual displacement of the nuclei, proportional in number to the infectivity of the inoculum.

The antigenicity of purified virus preparations is separable into two components, the one having virus specificity

and the other that of normal chicken tissue; whether the latter represents an integral part of the virus or contamination is not clear. It is of particular interest that some of the tar-induced tumors are immunologically related to the Rous sarcoma 1 virus; for example, pheasants inoculated with a nonfilterable tar-induced sarcoma produced antibody to the Rous virus. It has been reported also that certain carcinogen-induced tumors are subsequently transmissible by cell-free preparations.

REFERENCES

1. Anderson, W. A., Macgruder, B., and Kilbourne, E. D.: Induced reactivation of herpes simplex virus in healed rabbit corneal lesions. Proc. Soc. Exptl. Biol. Med., 1961, **107:**628–632.
2. Andervont, H. B.: Problems concerning the tumor viruses. In Burnet, F. M., and Stanley, W. M. (eds.): The Viruses, vol. 3, pp. 307–368. Academic Press, New York. 1959.
3. Andrewes, C. H.: Virus III in tissue cultures; appearance of intranuclear inclusions in vitro. Brit. J. Exptl. Path., 1929, **10:**188–190.
4. Appleyard, G.: An immunizing antigen from rabbit-pox and vaccinia viruses. Nature, 1961, **190:**465–466.
5. Armstrong, C., Daniels, W. B., MacMurry, F. G., and Turner, H. C.: Complement fixation in cat scratch disease employing Lygranum C. F. as antigen. J. Amer. Med. Assn., 1956, **161:**149–150.
6. Bachrach, H. L., and Breese, S. S., Jr.: Purification and electron microscopy of foot-and-mouth disease virus. Proc. Soc. Exptl. Biol. Med., 1958, **97:**659–665.
7. Bachrach, H. L., Callis, J. J., Hess, W. R., and Patty, R. E.: A plaque assay for foot-and-mouth disease virus and kinetics of virus reproduction. Virology, 1957, **4:**224–236.
8. Banfield, W. G., and Brindley, D. C.: An electron microscope study of the epidermal lesion of molluscum contagiosum. Ann. N. Y. Acad. Sci., 1959, **81:**145–163.
9. Banks, H. S.: Infection across the intact conjunctiva. Lancet, 1958, **ii:**518–519.
10. Bartell, P., and Tint, H.: Correlation of three potency assay methods for smallpox vaccines. J. Immunol., 1962, **88:**348–353.
11. Beard, J. W.: Etiology of avian leukosis. Ann. N. Y. Acad. Sci., 1957, **68:**473–485.
12. Beard, J. W., Sharp, D. G., and Eckert, E. A.: Tumor viruses. Adv. Virus Res., 1955, **3:** 149–197.
13. Black, F. L., Reissig, M., and Melnick, J. L.: Measles virus. Adv. Virus Res., 1959, **6:**205–230.
14. Blank, H., Coriell, L. L., and Scott, T. F. McN.: Human skin grafted onto the chorioallantois of the chick embryo for virus cultivation. Proc. Soc. Exptl. Biol. Med., 1948, **69:**341–345.
15. Blattner, R. J., and Heys, F. M.: Role of viruses in the etiology of congenital malformations. Progress Med. Virol., 1961, **3:** 311–362.
16. Brooksby, J. B.: The virus of foot-and-mouth disease. Adv. Virus Res., 1958, **5:**1–37.
17. Brown, A., Elsner, V., and Officer, J. E.: Growth and passage of variola virus in mouse brain. Proc. Soc. Exptl. Biol. Med., 1960, **104:**605–608.
18. Brown, J. A. H.: Studies on antigenic structure of herpes virus. Brit. J. Exptl. Path., 1953, **34:**290–299.
19. Brumfitt, W., and O'Grady, F.: Laboratory diagnosis of glandular fever. Postgraduate Med. J., 1958, **34:**535–540.
20. Cappell, D. F., and McFarlane, M. N.: Inclusion bodies (protozoon-like cells) in organs of infants. J. Path. Bact., 1947, **59:** 385–398.
21. Chaproniere, D. M.: The effect of myxoma virus on cultures of rabbit tissues. Virology, 1956, **2:**599–610.
22. Charles, A.: An electron microscope study of molluscum contagiosum. J. Hyg., 1960, **58:** 45–56.
23. Clemençon, G., and Kiebsch, I.: Beitrag zur Klinik der Mononucleosis infectiosa. Deut. med. Woch., 1958, **83:**257–276.
24. Collier, W. A., Smit, A. M., and van Heerde, A. F.: Der Nachweis von Antihämagglutininen bei Variolapatienten als diagnostisches Hilfsmittel. Ztschr. f. Hyg. u. Infektionskr., 1950, **131:**555–567.
25. Cross, R. M.: Observations on the classification and interpretation of reactions to smallpox vaccination. Bull. W. H. O., 1961, **25:**7–17.
26. Cutchins, E., and Warren, J.: Comparative susceptibility of cell cultures to vaccinia virus: Application to the standardization of smallpox vaccine. Proc. Soc. Exptl. Biol. Med., 1958, **97:**456–462.
27. Cutchins, E., Warren, J., and Jones, W. P.: The antibody response to smallpox vaccination as measured by a tissue culture plaque method. J. Immunol., 1960, **85:**275–283.
28. Dawe, C. J., Law, L. W., Morgan, W. D., and Shaw, M. G.: Morphologic responses to tumor viruses. Fed. Proc., 1962, **21:**5–14.
29. DeOme, K. B.: The mouse mammary tumor virus. Fed. Proc., 1962, **21:**15–18.
30. Dmochowski, L.: The milk agent in the origin of mammary tumors in hybrid mice. Adv. Cancer Res., 1953, **1:**103–172.
31. Dmochowski, L.: Viruses and tumors. Bact. Rev., 1959, **23:**18–40.
32. Dmochowski, L.: The viral etiology of leukemia. Progress Med. Virol., 1961, **3:**363–494.
33. Dmochowski, L.: Viruses and tumors. Science, 1961, **133:**551–561.
34. Dmochowski, L., Grey, C. E., Burmester, B. R., and Gross, M. A.: Submicroscopic morphology of avian neoplasms. III. Studies on visceral lymphomatosis. Proc. Soc. Exptl. Biol. Med., 1959, **100:**514–519.
35. Dmochowski, L., Grey, C. E., and Burmester, B. R.: Submicroscopic morphology of avian neoplasms. IV. Studies on erythroblastosis of strain RPL-12. Proc. Soc. Exptl. Biol. Med., 1959, **100:**517–519.
36. Dodd, M. C., Graber, C. D., and Anderson, G.: Hemagglutination of rabbit erythrocytes by pus from cases of cat scratch fever. Proc. Soc. Exptl. Biol. Med., 1959, **102:**556–558.

37. Downie, A. W.: Infection and immunity in smallpox. Lancet, 1951, **ii**:419–422.
38. Downie, A. W., et al.: Studies on the virus content of mouth washings in the acute phase of smallpox. Bull. W. H. O., 1961, **25**: 49–53.
39. Dumbell, K. R., Bedson, H. S., and Rossier, E.: The laboratory differentiation between variola major and variola minor. Bull. W. H. O., 1961, **25**:73–78.
40. Eddy, B. E.: Polyoma virus, Section B. Adv. Virus Res., 1960, **7**:91–102.
41. Enders, J. F., et al.: Measles virus: A summary of experiments concerned with isolation, properties and behavior. Amer. J. Pub. Health, 1957, **47**:275–282.
42. Epstein, M. A.: Observations on the Rous virus, fine structure and relation to cytoplasmic vacuoles. Brit. J. Cancer, 1957, **11**: 268–273.
43. Falke, D.: Isolation of two variants with different cytopathic properties from a single strain of herpes B virus. Virology, 1961, **14**: 492–495.
44. Fenner, F.: The clinical features and pathogenesis of mouse-pox (infectious ectromelia of mice). J. Path. Bact., 1948, **60**:529–552.
45. Fenner, F.: Mouse pox (infectious ectromelia of mice). A review. J. Immunol., 1949, **63**: 341–373.
46. Fenner, F.: The biological characters of several strains of vaccinia, cowpox and rabbitpox viruses. Virology, 1958, **5**:502–529.
47. Fenner, F., and Comben, B. M.: Genetic studies with mammalian pox-viruses. I. Demonstration of recombination between two strains of vaccinia virus. Virology, 1958, **5**:530–548.
48. Fenner, F., and Marshall, I. D.: A comparison of the virulence for European rabbits (*Oryctolagus cuniculus*) of strains of myxoma virus recovered in Australia, Europe and America. J. Hyg., 1957, **55**:149–191.
49. Fenner, F., and Woodroofe, G. M.: The pathogenesis of infectious myxomatosis: The mechanism of infection and the immunological response in the European rabbit (*Oryctolagus cuniculus*). Brit. J. Exptl. Path., 1953, **34**:400.
50. Frankel, J. W., and West, M. K.: Cultivation of measles virus in a stable line of human amnion cells. Proc. Soc. Exptl. Biol. Med., 1958, **97**:741–742.
51. Gaylord, W. H., and Melnick, J. L.: Intracellular forms of pox viruses as shown by the electron microscope (vaccinia, ectromelia, molluscum contagiosum). J. Exptl. Med., 1953, **98**:157–172.
52. Gildemeister, H., et al.: Praktikum der Virusdiagnostik. Gustav Fischer Verlag, Stuttgart. 1961.
53. Gillespie, J. H., and Karzon, D. T.: A study of the relationship between canine distemper and measles in the dog. Proc. Soc. Exptl. Biol. Med., 1960, **105**:547–551.
54. Goodpasture, E. W., and Anderson, K.: Infection of human skin, grafted on the chorioallantois of chick embryo, with the virus of herpes zoster. Amer. J. Path., 1944, **20**:447–455.
55. Gray, A., Tokimaru, T., and Scott, T. F. McN.: Different cytopathogenic effects observed in HeLa cells infected with herpes simplex virus. Arch. f. d. gesamte Virusf., 1958, **8**: 59–76.
56. Gross, L.: The aetiology of cancer and allied diseases. Development of a concept based on recent experiments dealing with a cell-free transmission of mouse leukemia. Brit. Med. J., 1958, **ii**:1–5.
57. Gross, L. (ed.): Oncogenic Viruses. Pergamon, New York. 1961.
58. Guyton, T. B., Ehrlich, F., Blanc, W. A., and Becker, M. H.: New observations on generalized cytomegalic inclusion disease of the newborn. New England J. Med., 1957, **257**: 803–807.
59. Habel, K.: Resistance of polyoma virus immune animals to transplanted polyoma tumors. Proc. Soc. Exptl. Biol. Med., 1961. **106**:722–725.
60. Hahon, N., and Wilson, B. J.: Pathogenesis of variola in *Macaca irus* monkeys. Amer. J. Hyg., 1960, **71**:69–80.
61. Harris, R. J. C.: Properties of the agent of Rous No. 1 sarcoma. Adv. Cancer Res., 1953, **1**:233–271.
62. Herrlich, A., and Mayr, A.: Die Pocken: Erreger, Epidemiologie und klinisches Bild. Georg Thieme Verlag, Stuttgart. 1960.
63. Hummeler, K., et al.: Encephalomyelitis due to infection with *Herpesvirus simiae* (herpes B virus). A report of two fatal, laboratory-acquired cases. New England J. Med., 1959, **261**:64–68.
64. Ingalls, T. H., Babbott, F. L., Jr., Hampson, K. W., and Gordon, J. E.: Rubella: Its epidemiology and teratology. Amer. J. Med. Sci., 1960, **239**:363–383.
65. Jawetz, E., Coleman, V. R., and Allende, M. F.: Studies on herpes simplex virus. II. A soluble antigen of herpes virus possessing skin reactive properties. J. Immunol., 1951, **67**:197–205.
66. Kaplan, A. S.: A study of the herpes simplex virus–rabbit kidney cell system by the plaque technic. Virology, 1957, **4**:435–457.
67. Kaplan, C., and Micklem, L. R.: A method for preparing smallpox vaccine on a large scale in cultured cells. J. Hyg., 1961, **59**:171–180.
68. Kaplan, H. S.: Possible mechanisms of carcinogenesis. Fed. Proc., 1962, **21**:1–4.
69. Katz, S. L., Milovanovic, M. V., and Enders, J. F.: Propagation of measles virus in cultures of chick embryo cells. Proc. Soc. Exptl. Biol. Med., 1958, **97**:23–29.
70. Kidd, J. G.: Proliferative lesions caused by viruses and virus-like agents. In Kidd, J. G. (ed.): The Pathogenesis and Pathology of Virus Diseases, pp. 161–180. Columbia University Press, 1950.
71. Kilham, L.: Propagation of fibroma virus in tissue cultures of cottontail testes. Proc. Soc. Exptl. Biol. Med., 1956, **92**:739–742.
72. Kilham, L.: The fibroma-myxoma transformation. Adv. Virus Res., 1960, **7**:103–129.
73. Kluge, R. C., Wicksman, R. S., and Weller, T. H.: Cytomegalic inclusion disease of the newborn. Report of a case with persistent viruria. Pediatrics, 1960, **25**:35–39.
74. Kolb, R. W., Cox, C. B., and Aylor, H. T.: Improvements in the rabbit scarification method for the assay of smallpox vaccine. Bull. W. H. O., 1961, **25**:19–24.
75. Kolb, R. W., Cutchins, E. C., Jones, W. P., and Aylor, H. T.: A comparison of the rabbit scarification technique with titrations in cell cultures for the potency assay of smallpox vaccine. Bull. W. H. O., 1961, **25**:25–32.
76. Krugman, S., Goodrich, C. H., and Ward, R.:

Primary varicella pneumonia. New England J. Med., 1957, **257:**843–848.
77. Krugman, S., and Ward, R.: Rubella problem; clinical aspects, risk of fetal abnormality and methods of prevention. J. Pediatrics, 1954, **44:**489–498.
78. Kunz, L. J.: Nonbacterial regional lymphadenitis ("cat-scratch fever"). New England J. Med., 1961, **265:**591–592.
79. Lebrun, J.: Cellular localization of herpes simplex virus by means of fluorescent antibody. Virology, 1956, **2:**496–510.
80. Leibowitz, S.: Infectious Mononucleosis. Grune and Stratton, New York. 1953.
81. Loh, P. H., and Riggs, J. L.: Demonstration of the sequential development of vaccinial antigens and virus in infected cells: Observations with cytochemical and differential fluorescent procedures. J. Exptl. Med., 1961, **114:**149–160.
82. Luse, S. A., and Smith, M. G.: Electron microscope studies of cells infected with salivary gland viruses. Ann. N. Y. Acad. Sci., 1959, **81:**133–144.
83. Manaker, R. A., and Groupé, V.: Discrete foci of altered chicken embryo cells associated with Rous sarcoma virus in tissue culture. Virology, 1956, **2:**838–840.
84. Marshall, R. G., and Gerone, P. J.: Susceptibility of suckling mice to variola virus. J. Bact., 1961, **82:**15–19.
85. Mayr, A., and Herrlich, A.: Züchtung des Variolavirus in der infantilen Maus. Arch. f. d. gesamte Virusf., 1960, **10:**226–235.
86. McCarthy, K., Downie, A. W., and Armitage, P.: The antibody response in man following infection with viruses of the pox group. I. An evaluation of the pock counting method for measuring neutralizing activity. J. Hyg., 1958, **56:**84–100.
87. Mellors, R. C.: Viruses, genes and cancer. Fed. Proc., 1958, **17:**714–723.
88. Melnick, J. L., et al.: Electron microscopy of viruses of human papilloma, molluscum contagiosum, and vaccinia, including observations of the formation of virus within the cell. Ann. N. Y. Acad. Sci., 1952, **54:**1214–1225.
89. Melnick, J. L., and Banker, D. D.: Isolation of B virus (herpes group) from the central nervous system of a rhesus monkey. J. Exptl. Med., 1954, **100:**181–194.
90. Melnick, J. L., Strauss, M. J., Bunting, H., and Shaw, E. W.: "Crystalline" virus-like bodies from human skin papillomas. J. Appl. Phys., 1950, **21:**70.
91. Mendelson, C. G., and Kligman, A. M.: Isolation of wart virus in tissue culture. Successful reinoculation into humans. Arch. Dermat., 1961, **83:**559–562.
92. Mermelstein, R. H., and Freireich, A. W.: Varicella pneumonia. Ann. Intern. Med., 1961, **55:**456–463.
93. Mikkelsen, W., Tupper, C. J., and Murray, J.: The ox cell hemolysin test as a diagnostic procedure in infectious mononucleosis. J. Lab. Clin. Med., 1958, **52:**648–652.
94. Moloney, J. B.: The murine leukemias. Fed. Proc., 1962, **21:**19–31.
95. Morgan, C., Ellison, S. A., Rose, H. M., and Moore, D. H.: Structure and development of viruses as observed in the electron microscope. I. Herpes simplex virus. J. Exptl. Med., 1954, **100:**195–202.
96. Moura, R. A., and Warren, J.: Subclinical infection of dogs by canine-adapted measles virus evidenced by their subsequent immunity to canine distemper virus. J. Bact., 1961, **82:**702–705.
97. Murray, L. H.: A world review of smallpox incidence. Bull. W. H. O., 1951, **4:**394–420.
98. Nizamuddin, M., and Dumbell, K. R.: A simple laboratory test to distinguish the virus of smallpox from that of alastrim. Lancet, 1960, **i:**68–69.
99. Osterhout, S., and Tamm, I.: Measurement of herpes simplex virus by the plaque technique in human amnion cells. J. Immunol., 1959, **83:**442–447.
100. Palm, C. R., and Black, F. L.: A comparison of canine distemper and measles viruses. Proc. Soc. Exptl. Biol. Med., 1961, **107:**588–590.
101. Pierce, E. C., Pierce, J. D., and Hull, R. N.: B virus: Its current significance. Description and diagnosis of a fatal case of human infection. Amer. J. Hyg., 1958, **68:**242–250.
102. Pitt, D. B.: Congenital malformations and maternal rubella: Progress report. Med. J. Australia, 1961, **1:**881–890.
103. Prier, J. E.: Cat-scratch fever. Ann. N. Y. Acad. Sci., 1958, **70:**650–667.
104. Rake, G., Shaffer, M. F., and Jones, H. P.: Studies on measles. III. The use of tissue culture in propagation of measles virus. J. Infect. Dis., 1941, **69:**65–69.
105. Reid, J. D.: Cat scratch disease. New Zealand Med. J., 1959, **58:**39–44.
106. Report: Reports on public health and medical subjects No. 101. Rubella and other virus infections during pregnancy. Ministry of Health (Great Britain). H. M. Stationery Office, London. 1960.
107. Roizman, B., and Roane, P. R., Jr.: A physical difference between two strains of herpes simplex virus apparent by sedimentation in cesium chloride. Virology, 1961, **15:**75–79.
108. Rosanoff, E. I.: Hemagglutination and hemadsorption of measles virus. Proc. Soc. Exptl. Biol. Med., 1961, **106:**563–567.
109. Rosen, L.: Hemagglutination and hemagglutination-inhibition with measles virus. Virology, 1961, **13:**139–141.
110. Rous, P.: Concerning the cancer problem. Amer. Scientist, 1946, **34:**329–358.
111. Rowe, W. P., et al.: Cytopathogenic agent resembling human salivary gland virus recovered from tissue cultures of human adenoids. Proc. Soc. Exptl. Biol. Med., 1956, **92:**418–424.
112. Rowe, W. P., Hartley, J. W., Cramblett, H. G., and Mastrota, F. M.: Detection of human salivary gland virus in the mouth and urine of children. Amer. J. Hyg., 1958, **67:**57–65.
113. Rubin, H.: Special interactions between virus and cell in the Rous sarcoma. Society for General Microbiology, Symposium No. 9, pp. 171–194. Cambridge University Press, 1959.
114. Rubin, H.: Response of cell and organism to infection with avian tumor viruses. Bact. Rev., 1962, **26:**1–13.
115. Sanford, K. K., Likely, G. D., Bryan, W. R., and Earle, W. R.: The infection of cells in tissue culture with Rous sarcoma virus. J. Nat. Cancer Inst., 1952, **12:**1317–1343.
116. Schaeffer, M., Robinson, R. Q., and Fodor, A. R.: New viruses and virus diseases of man. Ann. Rev. Microbiol., 1959, **13:**335–348.
117. Schmidt, N. J., and Lennette, E. H.: A color-

imetric neutralization test for herpes simplex, with observations on neutralizing and complement-fixing antibody levels in human sera. J. Immunol., 1961, **86:**137–145.

118. Schmidt, N. J., Lennette, E. H., and Shon, C. W.: A complement-fixing antigen for herpes simplex derived from chick-embryo tissue cultures. Amer. J. Hyg., 1960, **72:**59–72.
119. Schmidt, J. R., and Rasmussen, A. F., Jr.: Activation of latent herpes simplex encephalitis by chemical means. J. Infect. Dis., 1960, **106:**154–158.
120. Scott, T. F. McN., and McLeod, D. L.: Cellular responses to infection with strains of herpes simplex virus. Ann. N. Y. Acad. Sci., 1959, **81:**118–128.
121. Scott, T. F. McN., McLeod, D. L., and Tokumaru, T.: A biologic comparison of two strains of *Herpesvirus hominis*. J. Immunol., 1961, **86:**1–12.
122. Shaffer, M. F., Rake, G., Stokes, J., Jr., and O'Neil, G. C.: Studies on measles. II. Experimental disease in man and monkey. J. Immunol., 1941, **41:**241–257.
123. Siegel, M., and Greenberg, M.: Fetal death, malformation and prematurity after maternal rubella: Results of a prospective study, 1949–1958. New England J. Med., 1960, **262:**389–393.
124. Smith, M. G.: Propagation in tissue cultures of a cytopathogenic virus from human salivary gland virus (SGV) disease. Proc. Soc. Exptl. Biol. Med., 1956, **92:**424–430.
125. Smith, M. G.: The salivary gland viruses of man and animals (cytomegalic inclusion disease). Progress Med. Virol., 1959, **2:**171–202.
126. Smith, M. G., and Vellios, F.: Inclusion disease or generalized salivary gland virus infection. Arch. Path., 1950, **50:**862–884.
127. Stewart, S. E.: Polyoma virus, Section A. Adv. Virus Res., 1960, **7:**61–90.
128. Symposium: The cytopathology of virus infection. Ann. N. Y. Acad. Sci., 1959, **81:**1–214.
129. Symposium: Studies on an attenuated measles-virus vaccine. New England J. Med., 1960, **263:**153–184.
130. Symposium: Live measles virus vaccine. Amer. J. Pub. Health, 1962, **52:**Suppl. Pt. II, 64 pp.
131. Symposium: Viruses and neoplasia. Fed. Proc., 1962, **21:**1–36.
132. Syverton, J. T.: The pathogenesis of the rabbit papilloma-to-carcinoma sequence. Ann. N. Y. Acad. Sci., 1952, **54:**1126–1141.
133. Tomcsik, J.: Eine neue serologische Reaktion zur Diagnose der Mononucleosis infectiosa. Schweiz. med. Woch., 1960, **90:**1444–1446.
134. Turner, W., Bigley, N. J., Dodd, M. C., and Anderson, G.: Hemagglutinating virus isolated from cat scratch disease. J. Bact., 1960, **80:**430–435.
135. Wang, S.-P., and Grayston, J. T.: Goat blood cells in agglutination test for infectious mononucleosis. Proc. Soc. Exptl. Biol. Med., 1959, **101:**111–113.
136. Warren, J.: The relationship of the viruses of measles, canine distemper and rinderpest. Adv. Virus Res., 1960, **7:**27–60.
137. Warren, J., and Cutchins, E. C.: Immunization of man against measles: Potential vaccines and problems. Amer. J. Pub. Health, 1962, **52:**80–86.
138. Warwick, W. J., and Good, R. A.: Cat-scratch disease in Minnesota. III. Evaluation of the intradermal skin test to cat-scratch disease antigen. Amer. J. Dis. Children, 1960, **100:**241–247.
139. Webster, R. G.: The immunological relations of the contagious pustular dermatitis virus to the mammalian pox group. Australian J. Exptl. Biol. Med. Sci., 1958, **36:**267–274.
140. Weller, T. H.: Serial propagation in vitro of agents producing inclusion bodies derived from varicella and herpes zoster. Proc. Soc. Exptl. Biol. Med., 1953, **83:**340–346.
141. Weller, T. H., and Coons, A. H.: Fluorescent antibody studies with agents of varicella and herpes zoster propagated in vitro. Proc. Soc. Exptl. Biol. Med., 1954, **86:**789–794.
142. Weller, T. H., Hanshaw, J. B., and Scott, D. E.: Serologic differentiation of viruses responsible for cytomegalic inclusion disease. Virology, 1960, **12:**130–132.
143. Wheeler, C. E.: Herpes simplex virus. Characteristics of a strain which produces unusually large multinucleated giant cells in tissue culture. Arch. Dermat., 1960, **82:**391–397.
144. Wildy, P., Russell, W. C., and Horne, R. W.: The morphology of herpes virus. Virology, 1960, **12:**204–222.

Chapter 38

THE INFLUENZA AND RELATED VIRUSES, INCLUDING MUMPS VIRUS (MYXOVIRUSES); THE ADENOVIRUSES; AND OTHER RESPIRATORY VIRUSES

THERE IS A large group of infectious diseases of the respiratory tract, including coryza, febrile catarrh, influenza, pneumonia or pneumonitis, pharyngitis, laryngotracheobronchitis, etc., which are casually referred to as flu, grippe, croup, colds, and the like. Some are a consequence of bacterial infections, fungous infections, or infection with certain of the rickettsiae. A large and important segment of acute respiratory disease is, however, of viral or probable viral etiology.

The diseases produced by viruses, or other microorganisms, are only partially differentiated by their clinical or epidemiological character and are separable only on an etiological basis with its implications to diagnosis, immunity, and epidemiology. Thus epidemic influenza has a distinctive epidemiological character and is caused by one or another of the immunological varieties of the influenza virus. At the same time, the individual case of influenza is clinically indistinguishable from coryzas such as the common cold in which there is not only no evidence of the presence of influenza virus, but in some instances other viruses have been found in possible, or even probable, causal relation to the disease. Similarly, the clinical entity primary atypical pneumonia, or PAP, is of diverse etiology, and may be caused by fungi or rickettsiae or pleuropneumonia-like organisms as well as by more than one kind of virus.

Elucidation of the viral etiology of a portion of diseases of this kind was initiated with the isolation and characterization of influenza virus in 1933 through reproduction of the disease in the ferret. Subsequent development of more sophisticated tissue culture techniques, coupled with the availability of antibiotics to minimize the effects of bacterial contamination of specimens such as nasopharyngeal washings, while not affecting the proliferation of the virus, made possible wide application of this method and the isolation of a series of hitherto unknown viruses.[150] The extension of readily applicable serological methods of characterization and identification as a corollary to tissue culture methods, and the application of such

serological methods to the retrospective diagnosis of infections of this kind, allow estimation of the relative prevalence of such viruses in the host population. In consequence, a general pattern of viral infections of this kind is beginning to emerge,[131, 152] although many gaps remain and a considerable portion of undifferentiated acute respiratory disease, or ARD, remains of as yet unknown etiology.

Aside from respiratory disease caused by members of the psittacosis group of viruses discussed earlier, the viruses associated with this kind of disease fall into three groups. The influenza and related viruses make up a group having in common an agglutinative reaction with fowl or other erythrocytes and known as the myxoviruses; the group also includes the causative agents of clinically diverse disease such as mumps, Newcastle disease, and fowl plague. The second group is that of the adenoviruses, described in 1953, which resemble one another in immunological character and host range. The third group is one of miscellaneous viruses which may prove to be prototypes of as yet unknown groups of viruses.[79, 164]

THE INFLUENZA VIRUSES

Influenza[144] is a widespread disease of man occurring in sporadic interepidemic form, in periodic epidemics, and in pandemic form. Pandemics occurred in 1890 and in 1918–1919; in the latter the disease occurred all over the world, and the estimated number of deaths was over twenty-one million. The epidemic periodicity differs somewhat between the two main types of influenza virus (see below), and epidemics of greater or lesser extent occur at 2- to 3-year intervals in the case of influenza A and at 4- to 5-year intervals with influenza B. The epidemic periodicity appears to be due to the accumulation of susceptibles, and the subtraction of immunes by death, in the population, but pandemic influenza seems to be a result of antigenic changes in the virus of sufficient magnitude that there is little effective cross-immunity between the pre-existing and new antigenic types.

The prevalence of the infection is indicated by the occurrence of demonstrable serum antibody in practically all adults and in most children over 5 years of age. It is probable that many infections are subclinical, or mild and regarded as common colds, and that the viruses persist in interepidemic periods in the form of such infections and sporadic cases. There seems to be no unequivocal evidence of the existence of a chronic carrier state. Such persistence of these viruses perhaps contributes to the maintenance of observed antibody titers in the general population.

Morphology.[78, 162] The influenza viruses are morphologically indistinguishable and are, for the most part, spherical in shape and have a mean diameter of 80 to 100 mμ, but there is some evidence that certain strains of influenza B are slightly larger than influenza A strains. As described elsewhere (Chap. 3), on primary isolation in the embryonated egg, and often for the first few passages, the virus occurs as both spheres and filaments; both have virus activity, and there is some reason to believe that the spherical particles may differentiate from the filamentous forms. Spherical particles cut centrally in ultrathin sections are found to contain a central body 20 to 22 mμ in diameter which is surrounded by less dense material and a limiting membrane about 3 mμ thick.[108] Analysis of purified preparations has given a chemical composition of 60 to 70 per cent protein, 5 per cent carbohydrate, 20 to 30 per cent lipid, and about 1 per cent nucleic acid of the pentose type.

Toxin.[34, 90, 91, 104] Like certain other viruses, the influenza viruses are toxic

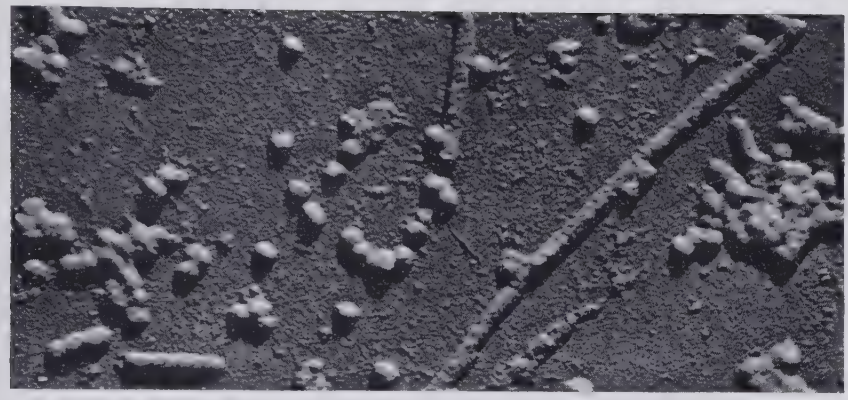


Figure 282. An electron micrograph of a dried suspension of partially purified influenza virus showing its rods and spheres of substantially constant diameters. × 16,000. (Wyckoff, J. Immunol.)

in high concentration, and the toxicity persists after appropriate inactivation, as by ultraviolet irradiation, but is destroyed before hemagglutinin and antigenic activity. The toxicity is demonstrable by intracerebral inoculation in the mouse with symptoms referable to meningo-encephalitic changes observed at autopsy. On intravenous or intraperitoneal inoculation the toxicity is pyrogenic in the rabbit, and fatal toxemia in the mouse is associated with liver and spleen damage with hyperemia, and focal necrosis in the former and destruction of the malpighian bodies in the latter. The toxicity may be neutralized by antiserum and is group-specific between influenza A and B with some strain differences observed. It is of some interest also that animals may be protected against the toxicity by pretreatment with receptor-destroying enzyme (see below), suggesting that the toxicity is related to attachment of the virus particle to the host cell.

Hemagglutinin.[5, 22] The affinity of the influenza and related viruses for mucinous substances on the surface of the erythrocyte and subsequent hemagglutination is the basis for the name myxovirus and their grouping together. The red cells of many species of animals are so agglutinated, and those of the chicken, man, and the guinea pig are usually used. Hemagglutination follows adsorption of the virus to the red cell, the attachment involving a mucoprotein receptor on the surface of the cell, and after a few hours the virus particles are spontaneously eluted. The eluted virus is capable of agglutinating fresh red cells, but the cells from which the virus has been eluted are not agglutinated by the same virus strain although they may be agglutinated by related viruses, and the order in which myxoviruses may be arranged in this respect is that of the so-called receptor gradient as described elsewhere (Chap. 3). There is reason to believe that the virus particle has an enzymatic activity which depolymerizes the receptor substrate, and the cells may be made inagglutinable by pretreatment with receptor-destroying enzyme, or RDE, present in cell-free filtrates of cultures of a number of kinds of bacteria, especially the cholera vibrio.

It is probable that adsorption of virus onto the erythrocyte is the test tube counterpart of the initial adsorption stage in the replication of the virus. Similar absorption and subsequent elution has been observed as occurring in the infected lung. Conversely, red cells are adsorbed by infected cells in tissue culture, the so-called hemadsorption phenomenon, and this reaction has been useful in the isolation of these viruses in tissue culture (see below). Following adsorption onto a susceptible cell, the virus enters the cell, proliferates, and is shed without cellular destruction as described elsewhere (Chap. 4).

The hemagglutinin activity is intimately associated with the virus particle but may be separated from it by ether

extraction. In fresh preparations the hemagglutinin activity parallels infectivity closely and is used as a measure of virus concentration. It is not, however, identical with infectivity; infectivity is destroyed at 42° C., leaving hemagglutinin activity unimpaired; after treatment at 56° C. the hemagglutinin is adsorbed on red cells but will not elute, and following treatment at 65° C. it is not adsorbed. The hemagglutinin of human strains is inactivated by trypsin, but that of swine influenza is not. The relative sensitivity of the human strains appears to be correlated with antigenic type[148] (see below).

Antigenic Structure.[85] Two kinds of antigens are present in these viruses. One is the soluble or S antigen which is found free in infected tissue, is also present in the virus particle from which it may be extracted, together with hemagglutinin, by ether, and is irregularly separable from the particle by sonic lysis. It is associated with a small particle, about 10 mμ in diameter and is

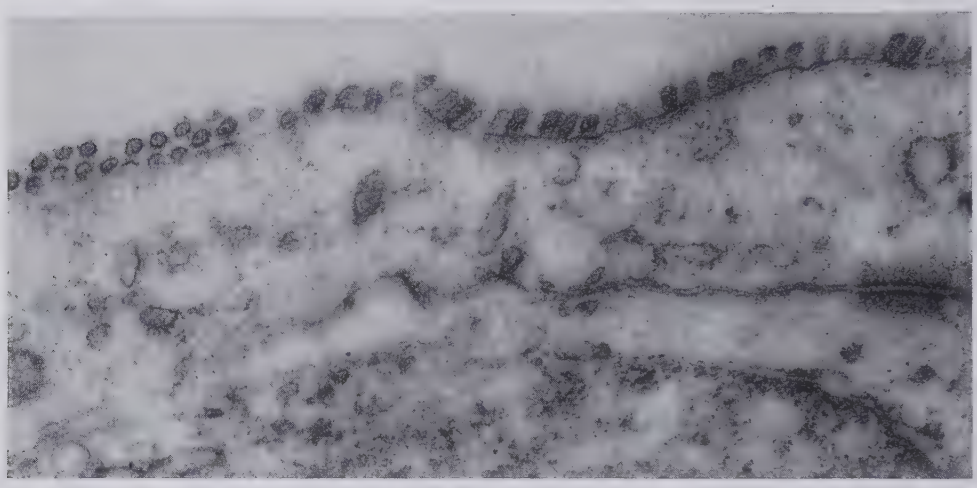


Figure 283. Electron micrograph of an ultrathin section of an entodermal cell of an influenza-infected chorioallantoic membrane. The virus particles in the form of spheres and short rods are on the surface of the cell, and the cell nucleus occupies the lower portion of the picture. × 45,000. (Morgan, Rose, and Moore, J. Exptl. Med.)

Figure 284. Electron micrograph of a cell similar to that shown in Figure 283. The structure of one spherical particle can be seen as consisting of an internal body, a sharply defined dense membrane, and an envelope of less dense material. The obliquely sectioned rod at the left appears to have an amorphous interior. × 152,000. (Morgan, Rose, and Moore, J. Exptl. Med.)

nucleoprotein in nature. It is type-specific and demonstrable by complement-fixation in the presence of antibody, and antibody to it is formed in response to infection, but not ordinarily in persons immunized with inactivated partially purified virus.

The second kind of antigen or V antigen is protein in nature and intimately associated with the virus particle. It contains multiple antigenicities and is strain-specific, the various antigenic components of the complex being demonstrable in varying proportions. Antibody to this kind of antigen is demonstrable as neutralizing or protective antibody, by complement-fixation and by the hemagglutination-inhibition, or HI, reaction. The last serological reaction is simple to carry out. The hemagglutinating unit (HU) of virus is determined by serial dilution and is represented by the highest dilution producing hemagglutination in 0.5 per cent erythrocyte suspension. Serial dilutions of the serum are titrated against four HU, and the end point is the highest dilution of serum that will prevent, or inhibit, the viral hemagglutination. Nonspecific, *i.e.*, non-antibody, inhibitors of a mucinous nature are often present in serum or tissue extracts, but only to low titer, and may be eliminated by pretreatment of the serum with RDE. Since the HI antibody is strain-specific and parallels, although it is not identical with, protective antibody, it is an extremely useful reaction for antibody surveys, is applicable to antigenic analysis of virus strains, etc.

Antigenic varieties. The influenza viruses are separable into at least three main types which are immunologically distinct from one another, *viz.*, type A which was isolated in 1933, type B isolated in 1940, and type C isolated in 1949. Each of these types is made up of strains, related to one another by the type specificity of the S antigen, but differing to a greater or lesser extent in the antigenic specificities contained in the V antigenic complex, and it has become almost axiomatic that a newly isolated strain will not be immunologically identical with strains isolated at other times. Such strain differences are demonstrable by the neutralization or protection test and by the HI reaction.

Type A influenza virus is the most common cause of influenza in man and shows the greatest strain variability. It has been possible, largely on the basis of the effectiveness of prophylactic immunity, to separate the multiplicity of type A strains into four main subgroups. The first of these is the swine influenza subgroup, strains of which were responsible for the 1918–1919 pandemic which persisted for perhaps 10 years as a cause of human influenza as indicated by serological evidence (see below). The first human strain to be isolated in 1933, the WS strain, is not identical with swine influenza but is more closely related to it than subgroups found later. The second subgroup is typified by the PR8 strain of virus. The human strains isolated in 1933 to 1935 were of this subgroup and were responsible for type A influenza for another 10 years. The third subgroup is the A-prime (A′) group, of which the FM1 strain is generally taken as a prototype. It appeared in 1946, and strains of this subgroup were responsible for human type A influenza through at least 1956.[32] The fourth subgroup is that of the Asian strains which first appeared in 1957.[107, 130] This time sequence in the appearance of antigenic subgroups is of particular significance in connection with naturally occurring antigenic variation (see below) and has practical consequences to effective prophylactic immunization.

Type B influenza viruses are also variable, but only two main subgroups are distinguished on the basis of the protective effect of immunization. The Lee strain is a prototype of the first of these, and Lee vaccine continued to give an effective prophylactic immunity until as late as 1954 although strains isolated somewhat earlier were found to be more distantly related to it. The second subgroup representing successors to the Lee subgroup has as its prototype the GL strains. Type C influenza viruses are less common than types A and B and appear

to be more stable in that, while strain differences are apparent, these are not sufficient for the separation of subgroups, and in general the type C viruses are relatively homogeneous. The type C viruses have not been associated with epidemic disease in man, and, in fact, their relation to clinical illness is somewhat obscure.

Variation.[73] There are three general kinds of variation among the influenza viruses, *viz.*, variation in hemagglutinin species specificity, variation in pathogenicity which perhaps includes hemagglutinin, and antigenic variation. There is also a variation between strains in their sensitivity to the nonspecific hemagglutinin inhibitor present in human and animal serums which appears to breed true even on continuous passage. On primary isolation the virus grows more readily in the amnionic than the allantoic cavity of the embryonated hen's egg and usually agglutinates fowl erythrocytes to a much lower titer than guinea pig or human O red cells, and the latter is expressed as the F:G hemagglutinin ratio. The primary isolate is said to be in the original, or O, state. After passage in the egg, the virus will grow in the allantoic cavity, is pathogenic for the mouse, and the F:G ratio approaches unity. Continued passage in the embryonated egg also often results in a rapid loss of virulence for man. When the virus shows these characteristics it is said to be in the derived, or D, state, and this change is the O-D variation which has its analogies with other microorganisms; the general phenomenon is discussed elsewhere (Chap. 7).

Other changes may be induced by continuous passage, *viz.*, the endotheliotropic variant produced by passage on the chorioallantoic membrane and the neurotropic variant resulting from passage in the mouse brain. As described elsewhere (Chap. 4), the inoculation of a nonadapted strain into the mouse brain results in an incomplete growth cycle with the appearance of noninfective or incomplete virus. There is also another kind of variation in hemagglutinin activity, characterized by partial or complete cross HI reactions in which three phases, designated P, Q, and R, may be distinguished, which seems to be a consequence of differences in avidity of the hemagglutinin for antibody.

Antigenic variation. Variation in the constituent antigens of the V complex occurs with some facility under natural conditions, and similar changes may be induced in the laboratory. When the subgroups are separated, as noted above, the naturally-occurring phenomenon appears to be a continuous variation in the antigenic character of type A virus, with occasional major changes of sufficient magnitude that there is no effective

Naturally Occurring Antigenic Variation Shown by Hemagglutination Inhibition*

	ANTISERUMS (FERRET)	RECIPROCAL OF TITERS**						
		TYPE A					TYPE B	
		swine 1930	WS 1933	PR8 1934	FM1 (A′) 1947	Asian 1957	Lee 1940	Todd 1950
type A	swine 1930	**4096**	48	<16	<16	<16	<16	<16
	WS 1933	32	**2048**	24	16	<16	<16	<16
	PR8 1934	<16	32	**4096**	24	<16	<16	<16
	FM1 1947	<16	<16	<16	**512**	<16	<16	<16
	Asian 1957	<16	<16	<16	<16	**256**	<16	<16
type B	Lee 1940	<16	<16	<16	<16	<16	**4096**	<16
	Todd 1950	<16	<16	<16	<16	<16	64	**4096**

* Compiled by Dr. Dorothy Hamre.

** Homologous titers are shown in bold face.

cross-immunity so far as man is concerned. The cross-reactions between major representative strains in the HI reaction are illustrated in the accompanying table. In the case of influenza B viruses, the second subgroup seems to have differentiated as a result of cumulative changes rather than a single major change. The evidence for this is of two kinds. One is the direct demonstration of antigenic differences in isolated strains, and the other is that of retrospective serological diagnosis in relation to age groups.

Surveys of serum antibody present in persons of all ages and sorted out with respect to specificity and age group reflect the prevalence in time of the subgroups of influenza A virus. In one such study completed in 1953, and later fully substantiated by studies both in this country and in England,[38, 39, 71] it was evident that the serum of children contained antibody to the then only recently prevalent A-prime strains; that of individuals in the 15 to 28 age group contained antibody to the PR8 subgroup as well; and antibody to the swine group, as well as to subsequently appearing antigenic varieties, was found only in persons at least 30 years of age. It is of special interest that, with the appearance of the Asian strains, antibody to this subgroup has been found in persons 70 to 90 years of age, suggesting that this apparently new antigenic variety may have been present in 1890.[36, 110, 111] The implication, of course, is that of the possibility of cyclic change, perhaps affected by human life span, rather than the appearance of new antigenic varieties in indefinite extension.

The validity of inferences as to a succession of antigenic varieties from the age distribution of serum antibody specificity is dependent upon the nature of the immune response in man.[70] It is suggested by data such as the foregoing, and substantiated experimentally by immunization with monovalent vaccines of representative antigenic varieties, that (a) the initial or childhood experience reflects the dominant antigens of the prevalent strains and is of limited effectiveness since the disease is most prevalent in this age group; (b) successive subsequent infections with differing antigenic varieties of the virus both reinforce the initial response and broaden it with the acquisition of new antibodies; and (c) the wide range of antibody activity in the serum of older persons is a consequence of contact with a broad range of influenza antigens with continuous reinforcement of prior antibody responses and the development of a progressively more effective immunity.

Antigenic variation may be produced under experimental conditions in a number of ways. Double infections with different virus strains result in recombination and the appearance of hybrids as well as parental types[20, 60, 73] (Chap. 7) in the same way that bacterial virus hybrids are produced, and similar changes have been produced in mixtures of active and inactive virus which appear to be analogous to the bacterial and fibroma-myxoma virus transductions.[14] Just as the antigenic structure of bacteria may be altered by growth in the presence of homologous antibody, influenza virus is similarly altered in the presence of antibody, both in egg culture[10, 11] and in infected partially immune animals,[55, 65, 66, 99] the changes in antigenic structure persisting in the absence of antibody. Representative changes produced by the latter method and demonstrable by the HI reaction

Experimentally Induced Antigenic Variation Shown by Hemagglutination-Inhibition*

ANTISERUMS (FERRET)	RECIPROCAL OF TITERS**		
	PARENT STRAIN (PR8)	SECOND VARIANT (Ba)	FIFTH VARIANT (Fd)
parent strain (PR8)	**1536**	48	8
second variant (Ba)	768	**1024**	128
fifth variant (Fd)	96	96	**768**

* Prepared by Dr. Dorothy Hamre.

* Homologous titers are shown in bold face.

are shown in the accompanying table. The conventional interpretation is that such antigenic variants arise from mutants and are selected by the antibody present. It is possible that some such mechanism is operative in the appearance of the naturally occurring antigenic variants of influenza virus.

Infection in Lower Animals. Animals other than man are not, so far as is known, naturally infected with the influenza viruses with the important exception of swine.

Swine influenza. This naturally-occurring respiratory disease was observed in swine in this country at the time of the influenza pandemic in 1918. The etiology of the disease was described by Shope in 1930 who isolated the swine influenza virus and a porcine variety of *Hemophilus influenzae* and showed that both kinds of microorganisms were required to produce the clinical disease. He later found a complex cycle of transmission, involving two reservoir hosts. The virus occurs in lungworms in infected swine, and the infected lungworm ova are excreted in the feces, ingested by earthworms, and the larvae develop in the earthworms. The infective cycle is completed when the infected earthworms are ingested by pigs, but the disease does not occur in the absence of some exciting cause, *H. influenzae* var. *suis* infection, but climatic conditions may apparently be effective also. The demonstration of the persistence of the infection in this masked form in intermediate hosts[142, 143] has been taken by some to suggest the possibility of the persistence of human influenza virus in some masked form, but there is no evidence to support such an inference.

There is little doubt that swine influenza virus is closely related to the influenza A virus strains prevalent during the 1918–1919 pandemic as indicated by serological studies such as those described above, but it seems likely that the animal infection was originally acquired from man.

Experimental infections. The ferret is highly susceptible to intranasal inoculation with the influenza viruses and may, in fact, be infected by contact. On primary isolation of the virus from man, there may be few symptoms of disease in the ferret, but the infection is demonstrable by a rise in antibody titer. On serial passage, virulence increases and there are overt symptoms of disease and lung consolidation. Similarly, the mouse lightly anesthetized with ether may be infected in primary isolation of the old type A strains by intranasal inoculation; fresh isolates usually do not produce lung lesions, but pulmonary consolidation is produced by passage strains of the virus. The A-prime and Asian strains are much less virulent for the mouse, and this animal is not practical for primary isolation of them. Type B influenza virus generally grows poorly in the mouse, even on prolonged passage. The embryonated hen's egg may be infected by various routes, but isolation is most often successful on inoculation of the amnionic cavity of 12- to 14-day old eggs. On passage the virus will grow in the allantoic cavity, in the yolk sac, and on the chorioallantoic membrane. Of these methods of isolation, inoculation of the amnionic cavity of the embryonated egg is regarded as one of the most sensitive.

The Disease in Man.[37] The virus enters the body via the respiratory tract, and the incubation period of influenza is short, 1 or 2 days, giving the epidemic and pandemic disease its explosive character. Symptoms referable to the respiratory tract, including nasal, pharyngeal, and laryngeal irritation and associated symptoms, occur but are often subordinate to the constitutional reaction with fever and prostration. The syndrome is variable and not characteristic, and influenza cannot be diagnosed on clinical grounds.

The most common complication, and usually the immediate cause of death in the fatal disease, is pneumonia resulting from secondary bacterial, most often staphylococcal, but also streptococcal or pneumococcal, infection.[72, 122, 140] Uncomplicated influenza may, though rarely, assume a fatal fulminating

form,[112, 132] and in such instances there is intense congestion of the trachea with consolidation and hemorrhage in the lungs, but little evidence of cellular reaction.

Immunity. Recovery from the disease results in the appearance of antibody to both S and V antigens giving complement-fixing, neutralizing, and HI reactions. Effective immunity is associated with antibody to the strain-specific V antigen. It persists for perhaps a few months, and there is evidence suggesting that it may be dependent upon the presence of neutralizing antibody in the respiratory mucosa.

The immunity resulting from initial exposure to the viral antigens appears to be of a low order but, as described above, seems to increase in effectiveness with repeated exposure to the virus so that the total experience of perhaps three decades provides a reasonable measure of effective immunity. This often, but not invariably, modifies subsequent infections so that the disease is subclinical or little more than a mild coryza. Such immunity is not effective against virus strains in which major antigenic changes have occurred.[86, 94]

Laboratory diagnosis.[96] The differentiation of influenza from a variety of clinically similar conditions is etiological and requires direct or indirect identification of the virus in the laboratory. Direct identification is accomplished most readily by the inoculation of nasal washings, containing appropriate antibacterial substances such as a mixture of penicillin and streptomycin, into the amnionic cavity of the 12- to 14-day old embryonated egg. Virus is present in the amnionic fluid in 2 to 4 days, may be detected by the agglutination of type O human erythrocytes, and identified by the HI reaction with known antiserum. The virus may also, but with less certainty, be isolated by intranasal inoculation of the ferret and subsequent demonstration of a rising antibody titer or the presence of the virus in the lung tissue.

The presence of the virus is shown indirectly by a rising antibody titer in paired serum specimens, the one taken as early as possible in the acute stage of the disease, and the other 10 days to 3 weeks later. The complement-fixation and HI reactions may be used; the former is less likely to be affected by minor antigenic differences between the test antigen and the infecting virus. A fourfold or greater rise in titer is regarded as diagnostic.

Prophylactic immunization. Prophylactic immunization against the disease is carried out with inactivated virus grown in the allantoic cavity of the embryonated egg and partially purified, commonly by adsorption and elution from chicken red cells. An appreciable degree of effective immunity against homologous or closely related strains of virus is apparent in about a week and persists for some months.[106] The degree of protection conferred is dependent upon the time elapsing between immunization and exposure, and it is estimated that 40 to 70 per cent of persons are protected.

Because of the changing antigenic character of virus strains, the vaccine strains are subject to alteration. Vaccine is usually multivalent, containing both type A and type B virus strains, but the type A has been changed from the PR8 strain to A-prime strains such as FM1, and later to include Asian strains, while the type B strains have shifted from the group having the Lee strain as a prototype to later-appearing types such as the GL or Todd strains. As prepared commercially, the vaccines are kept in monovalent form and mixed to give polyvalent vaccine of a composition determined by the nature of current isolates.

INFLUENZA-LIKE AND OTHER RESPIRATORY VIRUSES

A number of viruses other than the influenza viruses have been isolated from a variety of acute respiratory diseases lacking precise clinical characterization and considered to be common colds, pneumonitis, etc., and, on occasion, from apparently normal individuals. Some appear to be related to the influenza group, others make up the distinctive group of adenoviruses (see below), and still others are miscellaneous in the sense that they are unrelated to one another or to established groups of viruses. Antibody surveys have indicated, however, that they may be more prevalent than the frequency of isolation would suggest, and their occurrence emphasizes the diverse etiology of acute respiratory disease.[95]

PARAINFLUENZA VIRUSES

The viruses now considered to make up the parainfluenza group account for a considerable portion of ARD. Isolated from upper respiratory tract disease of varying severity, they account for much of such disease in children[25, 26, 30, 89] and are by no means unknown in adults.[19, 49] They are found in this country, in Europe,[145, 149] and in Australia[52] and are no doubt world-wide in occurrence. Although of varied etiology, the disease is substantially the same, resembling a common cold with pharyngitis, rhinitis, bronchitis, and fever, lasting 3 to 4 days,[114] and tending to be more severe in children. The etiological relation of these viruses to such upper respiratory disease is indicated not only by specific antibody response, but also by experimental reproduction of the disease in human volunteers.[157]

It has been observed that when guinea pig erythrocytes are added to monkey kidney cell cultures of influenza virus, the red cells are aggregated about and adsorbed by the cells in which viral proliferation is occurring.[141] This is not a unique property of myxoviruses for it occurs also with certain simian viruses and PPLO in cell culture.[17] Hemadsorption occurs prior to, or in the absence of, a cytopathic effect and is a sensitive indicator of the presence of myxovirus. Certain of the parainfluenza viruses isolated by application of this technique have been called hemadsorption, or HA, viruses. These and similar viruses, described under various names, and serologically distinct, or even unrelated, to one another and to the influenza viruses,[33] have been arranged as types of parainfluenza virus.[8]

Parainfluenza 1. This designation includes two viruses which are immunologically related but not identical, the Sendai virus and the HA 2 virus.

Sendai virus. (Newborn pneumonitis virus, hemagglutinating virus of Japan or HVJ, influenza virus type D). This virus, has been isolated repeatedly from both human and animal (swine, mice) sources in Japan since 1952. It appeared to be the etiological agent of highly fatal epidemic pneumonitis of newborn children in Japan and has been found in infants in Moscow[165] and in Germany.[146] It was also associated with an epidemic of influenza in Vladivostok in 1956, was isolated from affected individuals during the epidemic, and serum antibody to it persisted for some months in the local population.[56, 59]

The virus was originally isolated in the mouse lung where it produced influenza-like lesions, *e.g.*, consolidation, and is cultivable in the embryonated egg. It grows well in the allantoic cavity, and also on the chorioallantoic membrane where it produces small pock-like lesions or plaques similar to those produced by herpes simplex virus. It produces a hemagglutinin, agglutinating human, chicken, guinea pig, monkey, and mouse erythrocytes, and is serologically related to the influenza viruses, more closely to type B than the other types, and has been called influenza virus type D.

Significant antibody titers to the Sen-

dai virus have been observed in this country.[87] In convalescents elsewhere the HI titers reach levels of 1:32 to 1:64, the complement-fixing antibody titers 1:32, and the neutralizing antibody titers 1:128 to 1:795. In comparison, HI titers of 1:32 or higher were found in 52 per cent of 118 individuals under 18 years of age, and in 37 per cent of 248 individuals over 18 in this country, suggesting a relatively high prevalence of immunizing infection. The virus is, however, related to mumps virus[41] and some of the observed antibody titers may be a result of prior mumps infection. Fourfold or greater rises in HI antibody titer have also been observed in this country[42] in association with influenza-like disease and with a few cases of infectious mononucleosis.

HA 2 virus. Hemadsorption type 2 virus, together with HA 1, was isolated by the technique described above from children with febrile respiratory disease.[25] Essentially no cytopathic effect was apparent in monkey kidney cell culture, although hemadsorption occurred, but in later passages there was a partial cytopathic effect with the appearance of scattered round cells and eventually focal degeneration in the sheet. The culture fluid contains hemagglutinin, and complement-fixing antigen may be prepared from sonic lysate of the culture. Some of the strains will grow in the amnionic cavity of the 7-day embryonated hen's egg. This virus has also been isolated from adults[44] as well as repeatedly from children here[163] and in Japan.[53] Substantially the same virus has been isolated in Denmark[123, 124] where it was called Copenhagen 222. Infections with this virus account for a small portion of adult ARD, in one study[128] the figure given is 2.6 per cent, but it has been reported[95] that 84 per cent of American adults have antibody. The last is taken to suggest that the virus is widely disseminated. The independence of the Sendai and HA 2 strains is indicated by a lack of correlation in antibody found in surveys. The virus is not antigenically related to influenza, mumps, RS or ECHO 28 (see below) viruses. The pathogenicity of the cultured virus has been demonstrated by the inoculation of human volunteers to produce respiratory illness.[128]

Parainfluenza 2.[28] (croup associated or CA virus, acute laryngotracheobronchitis or ALTB virus.) This virus was isolated from two of twelve children suffering from acute laryngotracheobronchitis, or croup, by culture in monkey kidney tissue culture. A cytopathogenic effect appeared in 3 to 5 days and consisted of the formation of a syncytial mass with loss of cell boundaries and the appearance of vacuoles and granules in the cytoplasm. It has subsequently been isolated both in this country[92] and in England.[121]

This virus forms a hemagglutinin for chicken and human erythrocytes which has the unusual characteristic of producing agglutination at 4° C., but not at 37° C.; in fact, the process was repeatedly reversible by alternate warming and chilling. Red cells treated with RDE were inagglutinable, but after treatment with CA virus were still agglutinated by influenza viruses types A and B and by Newcastle disease virus. The virus particle was found to be 90 to 135 mμ in diameter.

In five of eleven children with the disease, a rise in antibody titer to this virus was observed. A small number of normal persons were examined, and significant titers of neutralizing antibody were found in four of sixteen individuals 34 months old or less and in nineteen of twenty individuals 21 to 30 years old. In contrast to the more or less constant prevalence of the other parainfluenza viruses, type 2 seems to be variable in its occurrence; 22 per cent of American adults are reported[95] to have antibody to it.

Parainfluenza 3. This virus was originally described as hemadsorption virus type 1, and was isolated, together with HA 2, from children having acute febrile respiratory disease.[40] It has subsequently been recovered from both children[35] and adults[103] and is apparently widely disseminated since antibody has been found in 100 per cent of adult Americans exam-

ined.[95] In cell culture the cytopathic effect results in the coalescense of adjacent cells to give multinucleated giant cells which eventually fall away to leave holes in the cell sheet.[40, 100, 101]

A hemadsorption virus was isolated[1, 4] from cattle suffering from shipping fever (which is also possibly of Pasteurella etiology [see Chap. 25]) and designated SF-4. This virus is serologically closely related to parainfluenza 3 virus strains of human origin. The relationship is so close that the two appear identical by the conventional serological methods, but small differences are apparent in the immune response of the guinea pig to intranasal inoculation.[2] Apparently the same bovine virus has been found in Sweden[12] and serological studies have indicated that the bovine infection is widespread with 70 per cent or more of cattle giving positive reactions.[3, 13] It is not known what relation there may be between the bovine and human diseases.

Other Myxoviruses. Other viruses, overtly similar, but of as yet uncertain status, have been described. An agent associated with human croup and designated U virus has been reported[125] which resembles parainfluenza 2 in that hemagglutination occurs more readily in the cold than at incubator temperatures. Another virus, the SA virus, has been isolated from hamster brain following inoculation with chick embryo allantoic culture of nasal washings from an individual with acute upper respiratory infection.[139] The hemagglutinating activity for guinea pig erythrocytes is similar to that of myxoviruses, and the cytopathic effect on monkey kidney culture is that of syncytial formation. It is apparently unrelated antigenically to other known viruses. Still another antigenically unrelated virus with the properties of a myxovirus was isolated[88] from a number of persons with upper respiratory illness in monkey kidney cell culture. The cytopathic effect is one of round cell formation, and the virus, designated M-25, is antigenically unrelated to the other myxoviruses. The virus described by French workers as EA 102 isolated from acute febrile respiratory illness in children[31] is characterized by a syncytial cytopathogenic effect in KB cell culture, and is serologically related to the HA viruses, but does not form a hemagglutinin. The status and significance of virus isolates such as the foregoing is as yet uncertain.

OTHER RESPIRATORY VIRUSES

Respiratory Syncytial (RS) Virus.[63, 102, 129, 154] (chimpanzee coryza agent, or CCA.) This virus was first isolated[109] from chimpanzees having symptoms of respiratory disease, and spontaneous infection occurred in a laboratory worker. The virus was grown from throat swabs on human liver tissue culture, with cytopathology apparent in about a week. On culture on human conjunctival cells or human embryo fibroblasts pathological changes were incomplete after 16 days of incubation. This virus did not infect the common laboratory animals. Both complement-fixing and neutralizing antibody were found in naturally and artificially infected chimpanzees and in thirteen of sixty-five persons over 10 years of age examined.

The virus has subsequently been isolated from children with bronchopneumonia and bronchitis rather than croup, both in this country[15, 29] and in England.[116] It did not grow on the chorioallantoic membrane of the embryonated egg, was not pathogenic for mice by the intracerebral route, and did not form a hemagglutinin. The virus particle was found to be 90 to 130 mμ in diameter, and the complement-fixing antigen smaller and separable from infectivity. Antibody to this virus was found in 80 per cent of children under 4 years of age examined. The cytopathology in tissue culture is that of syncytium formation, with loss of cell borders, and the presence of varying numbers of nuclei with altered chromatin pattern in the syncytial mass. This property of producing a syncytial type of cytopathology in tissue culture is shared with the measles virus.

ECHO 28 (2060-JH) Virus. This virus was isolated independently in two laboratories from individuals having mild upper respiratory disease. One group of strains from military recruits was designated by the prototype strain number as the 2060 virus,[119] and the other strains, isolated during two epidemics, were designated the JH virus.[126]

The virus was isolated in monkey kidney cell culture and is reported[105] to grow more readily in secondary than in primary cell culture. It is relatively difficult to work with and a plaque assay method has not been developed. It produces a degenerative type of cytopathic effect like that of many of the enteric viruses. It has not been grown in other cell cultures nor in the embryonated egg. No hemagglutinin is produced, a satisfactory complement-fixing antigen has not been prepared, and serological studies have been confined to neutralization in tissue culture.[77, 118] It has been found that the 2060 and JH strains are serologically identical, and the virus has been placed in the enterovirus group. (Chap. 39) as ECHO 28 with strain 2060 as the prototype.[117] It is not related serologically to the other enteric viruses, is not sensitive to ether, and is very small with an estimated size of 16 mμ.

The virus is not pathogenic for the conventional laboratory animals, including the suckling mouse and the monkey by the intracerebral route. Its pathogenicity for man is indicated by infectivity for human volunteers[82, 127, 155] to produce mild upper respiratory disease, and subsequent isolation from naturally infected young adults with the same symptoms.[67] Serological studies have suggested that the occurrence of antibody is relatively common in children as well as adults.

Eaton Agent. During the 1940's the disease known as primary atypical pneumonia, or PAP, was characterized as a clinical entity, but it became increasingly clear that this seeming entity may be of diverse etiology. In some instances the causal agent is psittacosis virus, in others it has been found to be adenovirus, and in still others the disease may be caused by such diverse microorganisms as Histoplasma, Coccidioides, or the rickettsiae of Q fever.

A portion of cases of PAP are set apart by the appearance of cold hemagglutinin which agglutinates homologous or type O human red cells at 4° C. but not at 37° C., and an agglutinin for the MG strain of nonhemolytic streptococcus.[6] The appearance of antibody to this strain of streptococcus is apparently coincidental for there seems to be no causal relation between this microorganism and the disease.

Eaton and his associates[47] described a viral agent in 1944 which could be cultivated in the chick embryo and which produced pneumonia in cotton rats and hamsters on intranasal inoculation. It was not until 1957 that the original strain was studied further, and additional, immunologically identical strains were isolated from a number of cases of the disease.[98] By means of the fluorescein-labeled antibody technique, it was shown that, following inoculation into the amnionic cavity of 13-day embryonated eggs, the agent multiplied exclusively in the cytoplasm of the epithelial cells lining the bronchioles and air sacs of the developing embryo.[45] The agent is relatively large, 150 to 250 mμ, and coccobacillary in shape. It is set apart from viruses other than those of the psittacosis group in that infections with it may be effectively treated with certain of the antibiotics,[46, 93] especially the tetracyclines. In 1962 it was cultivated by Chanock and his associates[27, 28] on lifeless mediums, first agar and subsequently broth, supplemented with 20 per cent horse serum and 2.5 per cent yeast extract. Its cultivability, susceptibility to tetracyclines, and other characteristics have identified it as a PPLO (Chap. 27) rather than a virus.

The Eaton agent appears to be an important cause of PAP, being responsible for as much as 90 per cent of cases of the disease showing cold agglutinin. It has been found repeatedly in all parts of this country. Its prevalence is indicated by serological

evidence of infection in 16 per cent of undiagnosed lower respiratory disease in a series of children, in 24 per cent of college students having nonbacterial pneumonic infections,[50] and in military personnel.[24]

THE COMMON COLD[7]

There is little or no doubt that the afebrile coryza designated the common cold is an infectious disease. The now classic study in Spitzbergen[115] clearly showed the significance of importation of an infectious agent into an isolated community to produce an epidemic of the common cold. The clinical syndrome defies precise definition and is often altered by secondary bacterial infection, but disease of this kind may be produced by a variety of agents, including bacteria, certain of the rickettsiae and fungi, and a number of established viruses such as the influenza virus as well as others not considered to be respiratory viruses. Such infections may be regarded as making up some portion of the common cold, or simulating the common cold, but the great bulk of disease of this kind remains unaccounted for in terms of a specific etiological agent or agents.

It seems probable that this large segment of respiratory disease is of virus etiology. Many workers, from the studies of Kruse in 1914 to the present time, have been able to reproduce the disease in human volunteers or chimpanzees by the inoculation of bacteria-free filtrates of nasal washings. Contemporary studies include the extensive investigations at the Common Cold Research Unit in Salisbury, England,[9] and similar work in this country, which have shown not only that the disease may be transmitted experimentally[83] but also that the agent present in filtered nasal washings is specifically neutralized by antibody present in pooled γ-globulin.[81] In the experimentally transmitted disease, the incubation period is short, 1 to 2 days, and the residual immunity on recovery is transient and apparently strain-specific.[80]

Attempts to culture viral agents responsible for respiratory disease characterized by coryza and associated symptoms have resulted in the isolation of a number of viruses such as those described above. Some of these viruses, such as the CCA and related forms, the 2060 group, and the JH virus, the Coe virus[97] which has been identified as Coxsackie A21, and certain of the adenoviruses, have been associated with respiratory disease in epidemic as well as sporadic form and may constitute a portion of the etiology of the common cold.

At the same time, a number of isolations of cold viruses have been described. In addition to the Salisbury strains, most recently, British workers[76, 156, 157, 158] have recovered a spectrum of such viruses in primary cell cultures of human fetal kidney which vary with respect to antigenicity, cytopathogenicity and cultivability. In this country, Hamparian, Ketler and Hilleman[64] have isolated a group of viruses from common cold infections, designated coryzavirus, in cell cultures derived from human fetal kidney and maintained in a diploid state. These viruses are not cultivable in conventional cell cultures and are not pathogenic for the usual laboratory animals. They are antigenically heterogeneous, *i.e.*, there are a number of serotypes, show no hemagglutination or hemadsorption, and are very small with an estimated diameter of 17 to 18 mμ. The status of these viruses with respect to the etiology of the common cold as distinct from mild upper respiratory disease of other, and presently known, etiology, remains to be determined.

THE MUMPS AND RELATED VIRUSES

The mumps virus, the Newcastle disease virus (NDV), and the virus of fowl plague are related to the influenza viruses with respect to hemagglutinin activity and affinity for the receptor substance of the erythrocyte and are regarded as members of the group of myxoviruses. In the receptor gradient order of inactivation of receptor sites on the red blood cell (Chap. 3), the mumps virus and NDV occur at one end of the series, *viz.*, red blood cells treated with mumps virus will still agglutinate with NDV and influenza A virus, those treated with NDV will agglutinate with influenza A virus, but not with mumps virus, and those treated with influenza A virus will no longer agglutinate with either mumps virus or NDV.

MUMPS

Mumps, or epidemic parotitis, is a highly contagious, widespread but seldom fatal disease of man that has long been known because of its distinctive clinical character, *e.g.*, swelling and tenderness of the parotid glands and orchitis in a variable but appreciable portion of cases. Some 60 per cent of adults are immune as compared with 90 per cent immune to measles virus. The disease tends to occur in the winter and early spring, with an epidemic periodicity of 7 to 8 years.

The mumps virus is present in the saliva and may be isolated within the first few days of the disease from the urine.[160, 161] It may be grown in a number of kinds of cell culture, the cytopathic effect differing with the virus strain and kind of cell culture, *e.g.*, on isolation in HeLa cell or monkey kidney cell culture foci of granular degeneration occur, but with adapted strains other effects are produced, varying with the type of cell culture.[61] Estimations of particle size have varied considerably; a size range commonly given is 90 to 135 mμ, but values as high as 340 mμ have been recorded. In electron micrographs the observed size of the spheroid particle varies, and it is probable that infectivity may be associated with particles of different size. It resembles the influenza viruses not only in hemagglutinating activity as noted above, but also its hemolytic activity in high virus concentration and the occurrence of two kinds of antigens. The S, or soluble, antigen is separable from the infectivity by differential centrifugation and fixes complement in the presence of antibody, and the V, or viral, antigen is found in the infective particle. Infectivity is destroyed by standing at room temperature for a few days or by 0.1 per cent formalin or ultraviolet irradiation with persistence of hemagglutinin and S and V antigenic activity, and the S antigen is relatively thermostable. The V antigen is related to that of the Sendai (parainfluenza 1) virus.[54]

Disease in Man. This disease occurs only in man, and there is no animal reservoir of infection, although several species of monkeys may be infected experimentally. The incubation period is about 3 weeks. A febrile reaction may or may not occur and is followed within 24 hours by enlargement of the salivary glands, usually the parotids but the sublingual and submaxillary glands may be affected also, and there is an edematous infiltration of the adjacent tissues. The swelling may be bilateral, unilateral, or unilateral followed by involvement of the unaffected side within a few days. It reaches a maximum in 48 hours and persists for 1 to 2 weeks, though some evidence of swelling may persist for longer periods. Orchitis occurs in perhaps 20 per cent of cases, but the proportion may vary considerably from one outbreak to another, and the involvement is bilateral in only 15 to 20 per cent.

Symptoms referable to the central nervous system, indicative of meningoencephalitis, occur in from less than 1 to

as much as 10 per cent of cases; such cases occurring in the absence of parotitis are often classed as aseptic meningitis. The pancreas, epididymis, prostate, and ovaries are affected only relatively rarely, although some degree of pancreatitis may be more common than generally thought. Involvement of tissues other than the salivary glands may occur prior to or after the parotitis, or even in the absence of parotitis. Epidemiological evidence, supported by a certain amount of experimental data, indicates that a considerable portion of infections may be asymptomatic but, as indicated above, the infection is less prevalent than measles.

Immunity. Strains of mumps virus appear to be of the same antigenic type, and recovery from the disease results in a solid and lasting immunity, possibly reinforced from time to time by reinfection, and unquestionable second attacks are quite rare. The immunity is demonstrable as complement-fixing, HI, and protective serum antibody and a hypersensitivity giving the delayed type of reaction to intradermal inoculation of inactivated virus.

The complement fixation is subject to refinement[51] if both S and V antigens are available because antibody to the S antigen appears earlier, within a few days of onset, and may reach high titer before antibody to V antigen is detectable. Antibody to the S antigen disappears more rapidly, leaving persisting antibody to the V antigen as evidence of past infection.

A fourfold or greater rise in complement-fixing antibody titer between paired serums, taken as early as possible in the disease and 2 to 3 weeks after onset, is regarded as diagnostic. Serological diagnosis is not important when the syndrome is typical but is of value in meningo-encephalitis without involvement of the salivary glands. The hypersensitivity appears too late in the disease to have diagnostic value and should, in fact, be avoided because the intradermal inoculation of inactivated virus often results in increased titers of antibody to the V antigen. Some convalescent serums give cross-reactions with NDV, and with Sendai virus.

There has been some interest in prevention or modification of the disease, especially with reference to orchitis in individuals past puberty, by the use of pooled γ-globulin or convalescent serum. Such passive immunization has, however, given only questionable results. Active immunization of monkeys and of man with egg culture virus inactivated with ether or ultraviolet radiation has been partially successful. In man a threefold difference in the incidence of the disease has been observed between immunized and control groups.[62, 69]

Experimental Infections. Mumps may be reproduced in several species of monkeys by direct inoculation of the gland via Stensen's duct and the infection passed in these animals by inoculation with suspensions of parotid gland tissue. The experimental disease is similar to that in man, with the occurrence of parotitis, facial edema, and associated pathology. Suckling mice and suckling hamsters may also be infected, and sufficient infection occurs in the lungs of adult hamsters to induce antibody formation. The virus may be grown in the embryonated hen's egg in the amnionic cavity and also in the yolk sac, growing more profusely after several passages; the virus is demonstrable by the appearance of hemagglutinin in the amnionic fluid whose specificity is determined by the HI reaction with known antiserum and complement-fixing antigen in the tissues of the amnionic sac. It also grows in monkey kidney tissue culture and in HeLa cell culture with cytopathogenic changes.

NEWCASTLE DISEASE VIRUS[68]

Newcastle disease (pseudo fowl pest, pseudo poultry plague, avian pest, avian distemper, avian pneumoencephalitis) is a disease of birds which is occasionally acquired by man through contact with infected birds. The natural hosts include domestic fowl such as chickens,

turkeys, guinea fowl, and parrots and wild birds such as sparrows and pigeons. There is some reason to believe that it may have originated in Indonesia where it was first described in 1926. It appeared in England in the same year and was eradicated by slaughter and terminal disinfection. It has since been reported from all over the world. It was first identified in this country in 1944 but may have been present as early as 1935. The virus tends to be pleomorphic and varies widely in its pathogenicity and tissue tropism, giving an impression that it may be evolving rapidly. It has been of particular interest as a viral agent that may become more closely adapted to man.

NDV. The virus particle is 80 to 120 mμ in diameter, but is not necessarily spherical, and may be filamentous or sperm-like in shape. The latter form has been reported as having a head 70 by 180 mμ and a tail as long as 500 mμ. It is unusually resistant under both natural and laboratory conditions. In the first instance, poultry quarters may remain infective for many weeks and produce disease in freshly introduced chickens. Under controlled conditions, some strains of the virus have been found to remain infective after exposure to 56° C. for as long as 180 minutes. In dried secretions and tissues viral activity persists for many months, and the virus has substantial resistance to disinfectants such as formalin and phenols, and glycol aerosols have failed entirely to prevent transmission of airborne infection.

The virus is readily cultivable in the embryonated egg and in several kinds of tissue cultures. When it is grown in the allantoic cavity, hemagglutinin is present in the allantoic fluid which falls between those of mumps virus and influenza A virus in the receptor gradient series noted above, and on this basis NDV is regarded as a member of the myxovirus group. Heavily infected allantoic fluid is also toxic, killing mice in 1 to 3 days after intravenous inoculation. Intranasal inoculation in this animal produces interstitial pneumonia that is a manifestation of toxicity for the virus does not multiply in the mouse lung.

Disease in Chickens. After an incubation period of 4 to 12 days, the onset of the disease in chickens is sudden and characterized by drowsiness, rapid respiration, and fever. Even before symptoms appear, the virus is widely distributed in the body, in highest concentration in the lungs and spleen. There is mucous discharge from the beak and nose, diarrhea, cyanosis, and respiratory distress, followed by convulsions and paralysis, with death in about a week. On autopsy multiple necrotic foci are found in the viscera, there are interstitial pneumonia, hemorrhagic patches in the gastrointestinal tract, and localized meningo-encephalitis with degeneration and necrosis and hemorrhage. This syndrome is subject to some variation, the respiratory and gastrointestinal symptoms being associated to some degree with Old World virus strains, while the pneumonic and encephalitic manifestations are often predominant in infection with American strains.

The disease is by no means invariably fatal, and, in fact, virulence varies widely between virus strains. Of two strains isolated in this country, for example, one killed 90 per cent or more of naturally infected chickens, while the other killed only 1 per cent. Under experimental conditions, the LD_{50} dose of cultured virus has been found to vary among strains from 0 to 10^8 per 0.5 ml. of allantoic fluid.

Diseased birds are highly infectious, shedding large amounts of virus in secretions and excretions. This, coupled with the resistance of the virus to drying, tends to favor the transmission of variants of the virus. The infection appears to be transmitted largely by aerosols of dried infectious material, but direct contact with diseased birds or infected carcasses may also be a source of infection. Although the virus is present in the blood, there is no evidence that biting arthopods transmit the infection.

Immunity. While there seem to be minor antigenic differences between

strains of the virus, chickens recovered from the disease are solidly immune to reinfection. Virus strains of low virulence are, in fact, used as immunizing agents, and are often administered as aerosols. The immunity is associated with the presence of serum antibody. It is titratable as complement-fixing antibody, neutralizing antibody antitoxic antibody, and HI antibody. Very high titers are found 2 to 3 weeks after initiation of the infection, and HI titers of 1:1280 and neutralizing antibody titers of 1:1 million are not uncommon.

Experimental infections. Guinea pigs, rabbits, and pigs are resistant to infection, the pneumonia produced in the mouse is not transmissible in series, but the ferret responds to intranasal inoculation with the production of antibody. Mice, hamsters, cotton rats, and rhesus monkeys may be infected by intracerebral inoculation to produce meningo-encephalitis of increasing severity with passage.

Disease in Man. Human infection is not common and appears to be confined almost exclusively to those who are closely associated with poultry, and laboratory infections have occurred. The incubation period is very short, 2 days or less, and the disease is a unilateral conjunctivitis without corneal involvement, and there may be pre-auricular adenitis, malaise, and chills, but without fever. No fatal cases have been reported, and recovery is complete within 2 weeks.

Serum antibody may or may not be detectable after recovery, and serodiagnosis is complicated by the presence of non-specific neutralizing activity in the serum. One of these is heat-labile and destroyed at 56° C. in 30 minutes, and the other is stable to such heating and associated with neutralizing antibody to mumps virus as noted above. It is supposed that at least some strains of mumps virus have antigens in common with NDV. In general, serodiagnosis of the human infection is not dependable.

The number of persons exposed to infection is very large. It is estimated that seven to eight million people are intimately associated with the raising and care of chickens in this country, and several hundred thousand others are concerned with the processing of poultry. All human cases found thus far have been initial infections acquired from infected birds, and transmission from man to man has not been observed. Neither has there been an instance of human infection acquired from dressed poultry. It is highly problematical whether strains of the virus of higher virulence for man may appear.

THE ADENOVIRUSES[20, 79]

The viruses known collectively as the adenoviruses were found in 1953, independently and almost simultaneously, by two groups of investigators. Rowe and his colleagues[135] cultured tonsil and adenoid tissue fragments removed at operation as explants in plasma clots in roller tube culture. On continued incubation, a degenerative cytopathology was produced which was transmissible in series in various kinds of tissue cultures, including HeLa cell, fibroblast, and epithelium tissue cultures. The cytopathogenic changes included the development of acidophilic swollen nuclei which appeared to contain inclusion bodies. The viruses found apparently exist in the form of latent infections in human adenoid or tonsil tissue.

In attempting to isolate viruses associated with acute respiratory disease, diagnosed in part as primary atypical pneumonia, Hilleman and Werner[75] cultured pharyngeal washings from affected individuals in HeLa cell culture. They isolated similar agents producing a degenerative cytopathology in tissue culture and showed that convalescent se-

rums contained significant titers of complement-fixing antibody to the viruses isolated.

Subsequently, similar viruses have been isolated all over the world. They form a group containing a common complement-fixing soluble antigen but fall into distinct serotypes by cross-neutralization tests in tissue culture or by complement-fixation with antiserums prepared in the rabbit.[120] This group of viruses was known first as the adenoid degenerative, or AD, viruses. When their relation to some kinds of pharyngitis and conjunctivitis became clear, they were called the adeno-pharyngeal-conjunctival, or APC, viruses. Subsequently it was generally agreed that these names would be dropped, and these viruses are now designated the adenoviruses.

Morphology and Growth. The biological and physical properties of these viruses are now well established. They will grow in a wide variety of tissue cultures, of which the HeLa cell culture is one of the most susceptible to infection. An unusual characteristic of growth in this kind of tissue culture is the development of an acid reaction in contrast to the alkaline reaction produced in human embryonic or monkey kidney tissue culture. They are not, however, cultivable in the embryonated hen's egg and are not pathogenic for the usual experimental animals.

The cytopathology in tissue culture includes morphological changes in the nuclei of infected cells which are described as a swelling of the nucleus, rearrangement of chromatin with accumulation of granular masses of basophilic material, the appearance of acidophilic masses, and wrinkling of the nuclear membrane. In cells infected with some strains of adenovirus, Feulgen-positive crystal-like structures are found which appear to correspond to the masses of particles found by electron microscopy.

The virus particles are 80 to 120 mμ in diameter, as determined by differential filtration of infectivity through graded collodion membranes, and in electron micrographs are spherical in shape. They occur in orderly arrays in masses of polyhedral shape or may be distributed at random in the cell. Occasionally doughnut shaped particles, having a hole in the center, are observed which are reminiscent of the incomplete phage particle (Chap. 4).

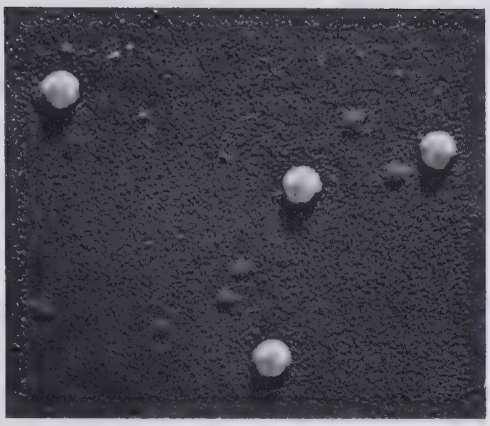


Figure 285. Electron micrograph of shadow-cast preparation of purified adenovirus. Note the dense center of the virus particles. × 17,000. (Touismis and Hilleman.)

These viruses are resistant to ether, but are inactivated by the usual disinfectants. They are stable over a relatively wide range in pH and are more stable than many viruses to standing at room temperature and at 36° C.; there is some difference between the serotypes in these respects, type 4, for example, persisting longer on standing, but being less stable at alkaline reactions than the other serotypes.

Antigenic Structure.[136] At least two kinds of antigens are present in these viruses which may be analogous to those found in the influenza and related viruses. One is the soluble, complement-fixing antigen which is associated with a smaller particle size, 25 to 40 mμ, than that of the virus particle, and is separable from infectivity. This antigen is group-specific, and gives cross-reactions between the serotypes.

Serotypes within the group are differentiable by neutralization tests carried out in tissue culture, and at least some are also separable by complement fixa-

tion, using antiserums prepared in the rabbit by immunization with virus grown in tissue culture immunologically heterologous to that used in the preparation of complement-fixing antigens.[18] A total of 28 differentiable serotypes have been described.[134] These types have been given Arabic numbers, and certain of them, types 3, 4, and 7, which have been examined in detail, have been found to be homogeneous even though the strains were widely different in temporal and geographical origin. Hemagglutinin is formed by many, but not all, types which is demonstrable with rhesus or rat erythrocytes, and the inhibition technique has been applied to the separation of serotypes.[133, 166]

Pathogenicity.[58, 113, 151] As indicated above, adenoviruses were discovered both as latent infections[48] in normal persons and in association with respiratory disease. The disease produced by these viruses is an acute infection of the mucous membranes of the respiratory tract and of the eye, with involvement of the submucous lymphadenoid tissues of these areas, including the regional lymph nodes. While it is probable that many of them have the capacity to produce similar disease, there is a definite association of certain serotypes with clinical syndromes, and the evidence that the association is causal is, in some instances, substantially complete.[137] Other serotypes, although found in association with disease, have not been shown to be specific etiological agents.

Acute respiratory disease. The adenovirus strains first isolated from military recruits with acute respiratory disease were type 4, and this serotype, together with types 3 and 7, has been found to be closely associated with the disease. The etiological relation of these serotypes to acute respiratory disease is indicated by a number of lines of evidence. In addition to repeated isolation from cases of such disease, rise in specific antibody titer occurs between paired serums from affected persons, and active immunization with polyvalent vaccine has been found to protect 50 to 70 per cent or better of nonimmunes exposed. Such vaccines have been prepared from formalin-inactivated virus grown in monkey kidney tissue culture. Further, serums from patients with acute respiratory disease, taken during World War II and stored in the interim, have been found to contain antibody to these serotypes, suggesting that the infection was prevalent at that time also.[74] Type 14 has been found to be similarly associated with acute respiratory disease in the Netherlands.

Types 1, 2, and 5, as well as some others, have been recovered repeatedly from tonsils and adenoids removed surgically from persons not ill with respiratory disease, but these types have been found in association with febrile respira-

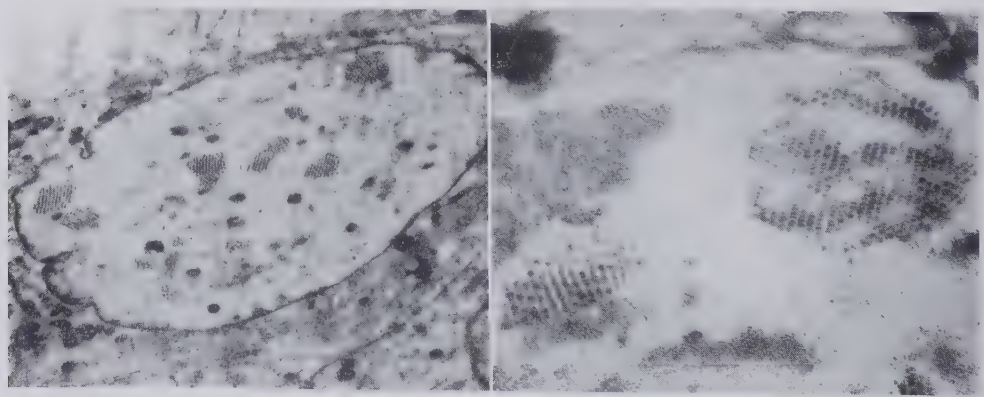


Figure 286. Electron micrographs of ultrathin sections of HeLa cells at 48 hours after infection with adenovirus type 4. *Left,* single particles and packed arrays of particles are distributed throughout the nucleus. *Right,* a higher magnification of a similar cell showing the single particles as spherical, ellipsoidal, or polygonal. (Touismis and Hilleman, Virology.)

tory infections occurring for the most part in infants.

Pharyngoconjunctival fever. This is a febrile disease characterized by pharyngitis and conjunctivitis which was defined as a clinical entity in connection with studies on adenoviruses. It occurs in epidemic form, largely in school children, and it is estimated that 50 per cent of nonimmunes exposed develop the disease. The conjunctivitis is usually unilateral, nonsuppurative, and does not result in corneal damage.

It appears to be definitely established that adenovirus type 3 is the specific etiological agent of this disease. The virus has been repeatedly isolated from patients with this disease, both from the throat and from the involved conjunctiva, but not from healthy persons. A rise in specific antibody titer occurs during the disease, and the conjunctival disease has been reproduced in human volunteers by instillation of virus into the eye.

Epidemic keratoconjunctivitis.[147] This is a highly infectious disease characterized by relatively little ocular exudate, the development of round subepithelial opacities in association with the keratitis, and often swelling of the regional lymph nodes. There may also be systemic symptoms, especially headache. The incidence of complications resulting in impairment of vision has been variable. The disease appeared in this country in 1941 and 1942 in epidemic form in shipyard workers on the West Coast and is thought to have been imported from Hawaii.

Adenovirus type 8 has been isolated repeatedly from this condition, in Japan, Italy, and Switzerland as well as in the United States. In one series of sixty cases studied,[84] 62 per cent had neutralizing serum antibody titers of 1:40 or higher and 87 per cent 1:20 or higher, with peak titers as high as 1:160. A fourfold or greater rise in antibody titer between paired serums was found in sixteen of eighteen cases of the disease. The evidence strongly suggests that this serotype of adenovirus is the etiological agent of this disease.

REFERENCES

1. Abinanti, F. R., et al.: Observations on infections of cattle with Myxovirus para-influenza 3. Amer. J. Hyg., 1960, **71:**52–58.
2. Abinanti, F. R., et al.: Relationship of human and bovine strains of Myxovirus para-influenza 3. Proc. Soc. Exptl. Biol. Med., 1961, **106:**466–469.
3. Abinanti, F. R., Hoerlein, A. B., Watson, R. L., and Huebner, R. J.: Serologic studies of Myxovirus para-influenza 3 in cattle and the prevalence of antibodies in bovines. J. Immunol., 1961, **86:**505–511.
4. Abinanti, F. R., and Huebner, R. J.: The serological relationships of strains of parainfluenza 3 virus isolated from humans and cattle with respiratory disease. Virology, 1959, **8:**391–394.
5. Anderson, S. G.: Hemagglutination by animal viruses. In Burnet, F. M., and Stanley, W. M. (eds.): The Viruses, vol. 3, pp. 21–50. Academic Press, New York. 1959.
6. Anderson, T. B.: Pneumonia with antibodies to streptococcus MG. Lancet, 1960, **i:**1375–1378.
7. Andrewes, C. H.: The common cold. Brit. Med. J., 1953, **i:**206–207.
8. Andrewes, C. H., Bang, F. B., Chanock, R. M., and Zhdanov, V. M.: Para-influenza viruses 1, 2 and 3: Suggested names for recently described myxoviruses. Virology, 1959, **8:** 129–130.
9. Andrewes, C. H., Lovelock, J. E., and Sommerville, T.: Experiment on transmission of colds. Lancet, 1951, **i:**25–27.
10. Archetti, I.: Further studies on antigenic variation of influenza A virus. Proc. Soc. Exptl Biol. Med., 1952, **80:**212–214.
11. Archetti, I., and Horsfall, F. L., Jr.: Persistent antigenic variation of influenza A viruses after complete neutralization in ovo with heterologous immune serum. J. Exptl. Med. 1950, **92:**441–462.
12. Bakos, K., and Dinter, Z.: Identification of a bovine mucosal-disease virus isolated in Sweden as *Myxovirus para-influenza* 3. Nature, 1960, **185:**549–550.
13. Bakos, K., and Dinter, Z.: Antikörperreaktion des Rindes auf die Infektion mit dem Virus der Parainfluenza 3. Zent. f. Bakt., I Abt. Orig., 1960, **180:**1–11.
14. Baron, S., and Jensen, K. E.: Evidence for genetic interaction between noninfectious and infectious influenza A viruses. J. Exptl. Med., 1955, **102:**677–697.
15. Beem, M., et al.: Association of the chimpanzee coryza agent with acute respiratory disease in children. New England J. Med., 1960, **263:**523–530.
16. Bell, J. A., et al.: Pharyngoconjunctival fever —epidemiological studies of a recently recognized disease entity. J. Amer. Med. Assn., 1955, **157:**1083–1092.
17. Berg, R. B., and Frothingham, T. E.: Hemadsorption in monkey kidney cell cultures of Mycoplasma (PPLO) recovered from rats. Proc. Soc. Exptl. Biol. Med., 1961, **108:** 616–618.
18. Binn, L. N., Hilleman, M. R., Rodriguez, J. E., and Glabere, R. R.: Antigenic relationships among adenoviruses with appraisal of reliability of complement-fixation tests for

typing isolates. J. Immunol., 1958, **80:**501–508.
19. Bloom, H. H., Johnson, K. M., Jacobsen, R., and Chanock, R. M.: Recovery of parainfluenza viruses from adults with upper respiratory illness. Amer. J. Hyg., 1961, **74:**50–59.
20. Brandon, F. B., and McLean, I. W., Jr.: Adenovirus. Adv. Virus Res., 1962, **9:**157–194.
21. Brandt, C. D.: Cytopathic action of myxoviruses on cultivated mammalian cells. Virology, 1961, **14:**1–10.
22. Buzzell, A., and Hanig, M.: Mechanism of hemagglutination by influenza viruses. Adv. Virus Res., 1958, **5:**290–346.
23. Chanock, R. M.: Association of a new type of cytopathogenic myxovirus with infantile croup. J. Exptl. Med., 1956, **104:**555–576.
24. Chanock, R. M.: Eaton agent pneumonia. J. Amer. Med. Assn., 1961, **175:**213–220.
25. Chanock, R. M., et al.: Newly recognized myxoviruses from children with respiratory disease. New England J. Med., 1958, **258:** 207–213.
26. Chanock, R. M., et al.: Association of hemadsorption viruses with respiratory illness in childhood. J. Amer. Med. Assn., 1959, **169:** 548–553.
27. Chanock, R. M., et al.: Growth of Eaton PPLO in broth and preparation of complement fixing antigen. Proc. Soc. Exptl. Biol. Med., 1962, **110:**884–889.
28. Chanock, R. M., Hayflick, L., and Barlie, M. F.: Growth on artificial medium of an agent associated with atypical pneumonia and its identification as a PPLO. Proc. Nat. Acad. Sci., 1962, **48:**41–49.
29. Chanock, R. M., Roizman, B., and Myers, R.: Recovery from infants with respiratory illness of a virus related to chimpanzee coryza agent (CCA). I. Isolation, properties and characterization Amer. J. Hyg., 1957, **66:** 281–290.
30. Chanock, R. M., Wong, D. C., Huebner, R. J., and Bell, J. A.: Serologic response of individuals infected with para influenza viruses. Amer. J. Pub. Health, 1960, **50:** 1858–1865.
31. Chany, C., et al.: Isolement et étude d'un virus syncytial non identifié associé à des affections respiratoires aigues du nourrisson. Ann. Inst. Pasteur, 1958, **95:**721–731.
32. Collins, S. D., and Lehmann, J. L.: Influenza epidemics during 1951–56 with a review of trends. Pub. Health Repts., 1957, **72:**771–780.
33. Cook, M. K., et al.: Antigenic relationships among the "newer" myxoviruses (parainfluenza). Amer. J. Hyg., 1959, **69:**250–264.
34. Cox, H. R.: Viral and rickettsial toxins. Ann. Rev. Microbiol., 1953, **7:**197–218.
35. Cramblett, H. G., Kasel, J. A., and Utz, J. P.: Respiratory illness in children simultaneously infected with hemadsorption virus type 1 and a newly recognized HeLa enterovirus (HE). Pediatrics, 1959, **24:**234–240.
36. Davenport, F. M.: Role of the Commission on Influenza. Studies of epidemiology and prevention. Pub. Health Repts., 1958, **73:**133–139.
37. Davenport, F. M.: Pathogenesis of influenza. Bact. Rev., 1961, **25:**294–300.
38. Davenport, F. M., Hennessy, A. V., and Francis, T., Jr.: Epidemiologic and immunologic significance of age distribution of antibody to antigenic variants of influenza virus. J. Exptl. Med., 1953, **98:**641–656.
39. Davenport, F. M., Stuart-Harris, C. H., Hennessy, A. V., and Francis, T., Jr.: Epidemiology of influenza. Comparative serological observations in England and the United States. Lancet, 1955, **i:**469–474.
40. Deibel, R., and Hotchin, J.: Growth characteristics of hemadsorption virus type 1 (*Myxovirus parainfluenza* 3) in tissue culture. Virology, 1961, **14:**66–73.
41. DeMeio, J. L., and Walker, D. L.: Demonstration of antigenic relationship between mumps virus and hemagglutinating virus of Japan. J. Immunol., 1957, **78:**465–471.
42. DeMeio, J. L., and Walker, D. L.: Sendai virus antibody in acute respiratory infections and infectious mononucleosis. Proc. Soc. Exptl. Biol. Med., 1958, **98:**453–458.
43. Dick, E. C., and Mogabgab, W. J.: Characteristics of para-influenza 1 (HA-2) virus. II. Hemagglutination, hemagglutination-inhibition and neutralization. Amer. J. Hyg., 1961, **73:**273–281.
44. Dick, E. C., Mogabgab, W. J., and Holmes, B.: Characteristics of para-influenza 1 (HA-2) virus. I. Incidence of infection and clinical features in adults. Amer. J. Hyg., 1961, **73:** 263–272.
45. Donald, H. B., and Liu, C.: Cytological studies of chick embryo cells infected with the virus of primary atypical pneumonia. Virology, 1959, **9:**20–29.
46. Eaton, M. D., and Liu, C.: Studies on the sensitivity to streptomycin of the atypical pneumonia agent. J. Bact., 1957, **74:**784–787.
47. Eaton, M. D., Meiklejohn, G., and van Herick, W.: Studies on the etiology of primary atypical pneumonia. A filterable agent transmissible to cotton rats, hamsters and chick embryos. J. Exptl. Med., 1944, **79:**649–668.
48. Evans, A. S.: Latent adenovirus infections of the human respiratory tract. Amer. J. Hyg., 1958, **67:**256–266.
49. Evans, A. S.: Infections with hemadsorption virus in University of Wisconsin students. New England Med. J., 1960, **263:**233–237.
50. Evans, A. S., and Brobst, M.: Bronchitis, pneumonitis and pneumonia in University of Wisconsin students. New England J. Med., 1961, **265:**401–409.
51. Fazekas de St. Groth, S., Graham, D. M., and Jack, I.: The serology of mumps infections. I. A new source of antigen and a simplified complement fixation test. J. Lab. Clin. Med., 1958, **51:**883–896.
52. Ferris, A. A.: The croup viruses: Bacteriological aspects. Med. J. Australia, 1960, **2:**768–769.
53. Fukumi, H. et al.: An epidemic due to HA2 virus in an elementary school in Japan. Japanese J. Med. Sci. Biol., 1959, **12:**307–317.
54. Gardner, P. S.: A serological relationship between mumps and Sendai viruses. J. Hyg., 1960, **58:**283–289.
55. Gerber, P., Hamre, D., and Loosli, C. G.: Antigenic variants of influenza A virus (PR8 strain). II. Serological and immunological characteristics of variants derived from variants. J. Exptl. Med., 1956, **103:**413–424.
56. Gerngross, O. G.: Peculiarities of the 1956

influenza outbreak in Vladivostok due to D virus. Problems Virol., 1957, **2**:71–75. (Translated from the Russian.)

57. Gildemeister, H., et al.: Praktikum der Virusdiagnostik. Gustav Fischer Verlag, Stuttgart. 1961.
58. Ginsberg, H. S.: Newer aspects of adenovirus infections. Amer. J. Pub. Health, 1959, **49**: 1480–1485.
59. Gorbunova, A. S., Gerngross, O. G., Gnorizova, V. M., and Bukrinskaia, A. G.: Strains of influenza virus of type D isolated in Vladivostok, and their role in the aetiology of the 1956 outbreak. Problems Virol., 1957, **2**:76–84. (Translated from the Russian.)
60. Gotlieb, T., and Hirst, G. K.: The experimental production of combination forms of virus. VI. Reactivation of influenza viruses after inactivation by ultraviolet light. Virology, 1956, **2**:235–248.
61. Gresser, I., and Enders, J. F.: Cytopathogenicity of mumps virus in cultures of chick embryo and human amnion cells. Proc. Soc. Exptl. Biol. Med., 1961, **107**:804–807.
62. Habel, K.: Vaccination of human beings against mumps: Vaccine administered at the start of an epidemic. I. Incidence and severity of mumps in vaccinated and control groups. Amer. J. Hyg., 1951, **54**:295–311.
63. Hamparian, V. V., et al.: Studies on acute respiratory illness caused by respiratory syncytial virus. I. Laboratory findings in 109 cases. Proc. Soc. Exptl. Biol. Med., 1961, **106**:717–722.
64. Hamparian, V. V., Ketler, A., and Hilleman, M. R.: Recovery of new viruses (coryzavirus) from cases of common cold in human adults. Proc. Soc. Exptl. Biol. Med., 1961, **108**:444–453.
65. Hamre, D., Loosli, C. G., and Gerber, P.: Antigenic variants of influenza A virus (PR8 strain). III. Serological relationships of a line of variants derived in sequence in mice given homologous vaccines. J. Exptl. Med., 1958, **107**:829–844.
66. Hamre, D., Loosli, C. G., and Gerber, P.: Antigenic variants of influenza A virus (PR8 strain). IV. Serological characteristics of a second line of variants developed in mice given polyvalent vaccine. J. Exptl. Med., 1958, **107**:845–855.
67. Hamre, D., and Procknow, J.: Virological studies on acute respiratory disease in young adults. Proc. Soc. Exptl. Biol. Med., 1961, **107**:770–773.
68. Hanson, R. P., and Brandly, C. A.: Newcastle disease. Ann. N. Y. Acad. Sci., 1958, **70**: 585–597.
69. Henle, W., et al.: Studies on the prevention of mumps. VII. Evaluation of dosage schedules for inactivated mumps vaccine. J. Immunol., 1959, **83**:17–28.
70. Hennessy, A. V., and Davenport, F. M.: Epidemiological implications of the distribution by age of antibody response to experimental influenza virus vaccines. J. Immunol., 1958, **80**:114–121.
71. Hennessy, A. V., Davenport, F. M., and Francis, T., Jr.: Studies of antibodies to strains of influenza virus in persons of different ages in sera collected in a postepidemic period. J. Immunol., 1955, **75**:401–409.
72. Hers, J. F. P., et. al.: Bacteriology and histopathology of the respiratory tract and lungs in fatal Asian influenza. Lancet, 1958, **ii**:1141–1143.
73. Hilleman, M. R.: Antigenic variation of influenza viruses. Ann. Rev. Microbiol., 1954, **8**:311–352.
74. Hilleman, M. R.: Epidemiology of adenovirus respiratory infections in military recruit populations. Ann. N. Y. Acad. Sci., 1957, **67**:262–272.
75. Hilleman, M. R., and Werner, J. H.: Recovery of new agent from patients with acute respiratory illness. Proc. Soc. Exptl. Biol. Med., 1954, **85**:183–188.
76. Hitchcock, G., and Tyrrell, D. A. J.: Some virus isolations from common colds. II. Virus interference in tissue cultures. Lancet, 1960, **i**:237–239.
77. Holper, J. C., et al.: Further studies on multiplication, serology and antigenicity of 2060 and JH viruses. J. Infect. Dis., 1960, **107**: 395–401.
78. Horne, R. W., Waterson, A. P., Wildy, P., and Farnham, A. E.: The structure and composition of the myxoviruses. I. Electron microscope studies of the structure of myxovirus particles by negative staining techniques. Virology, 1960, **11**:79–98.
79. Huebner, R. J., Rowe, W. P., and Chanock, R. M.: Newly recognized respiratory tract viruses. Ann. Rev. Microbiol., 1958, **12**:49–76.
80. Jackson, G. G., et al.: Susceptibility and immunity to common upper respiratory viral infections—the common cold. Ann. Intern. Med., 1960, **53**:719–738.
81. Jackson, G. G., Dowling, H. F., and Anderson, T. O.: Neutralization of common cold agents in volunteers by pooled human globulin. Science, 1958, **128**:27–28.
82. Jackson, G. G., Dowling, H. F., and Mogabgab, W. J.: Infectivity and interrelationships of 2060 and JH viruses in volunteers. J. Lab. Clin. Med., 1960, **55**:331–341.
83. Jackson, G. G., Dowling, H. F., Spiesman, I. G., and Boand, A. V.: Transmission of the common cold to volunteers under controlled conditions. I. The common cold as a clinical entity. Arch. Intern. Med., 1958, **101**:267–278.
84. Jawetz, E., Hanna, L., Kimura, S. J., and Thygeson, P.: Clinical diseases of adults associated with sporadic infection by APC viruses. Arch. Intern. Med., 1956, **98**:71–79.
85. Jensen, K. E.: The nature of serological relationships among influenza viruses. Adv. Virus Res., 1957, **4**:279–310.
86. Jensen, K. E., Dunn, F. L., and Robinson, R. Q.: Influenza, 1957. A variant and the pandemic. Progress Med. Virol., 1958, **1**:165–209.
87. Jensen, K. E., Minuse, E., and Ackermann, W. W.: Serologic evidence of American experience with newborn pneumonitis virus (type Sendai). J. Immunol., 1955, **75**:71–77.
88. Johnson, K. M., Chanock, R. M., Cook, M. K., and Huebner, R. J.: Studies of a new human hemadsorption virus. I. Isolation, properties and characterization. Amer. J. Hyg., 1960, **71**:81–92.

89. Kapikian, A. Z. et al. A study of the hemadsorption viruses (parainfluenza) and other viruses in children with and without respiratory disease. Pediatrics, 1960, **26:**243–248.
90. Kato, N., and Hara, H.: The toxic effect on rabbits of influenza virus given intravenously. Brit. J. Exptl. Path., 1961, **42:**145–152.
91. Kato, N., and Okada, A.: The relation of the toxic agent to the subunits of influenza virus particles. Brit. J. Exptl. Path., 1961, **42:**253–265.
92. Kim, H. W., Vargosko, A. J., Chanock, R. M., and Parrott, R. H.: Para-influenza 2 (CA) virus: Etiological association with croup. Pediatrics, 1961, **28:**614–621.
93. Kingston, J. R., et al.: Eaton agent pneumonia. J. Amer. Med. Assn., 1961, **176:**118–123.
94. Langmuir, A. D.: Asian influenza in the United States. Ann. Intern. Med., 1958, **49:**483–492.
95. La Placa, M., and Moscovici, C.: Distribution of parainfluenza antibodies in different groups of population. J. Immunol., 1962. **88:**72–77.
96. Lennette, E. H.: Laboratory diagnosis of influenza by virus isolation. Amer. Rev. Resp. Dis., 1961, **83:**116–119.
97. Lennette, E. H., Fox, V. L., Schmidt, N. J., and Culver, J. O.: The Coe virus: An apparently new virus recovered from patients with mild respiratory disease. Amer. J. Hyg., 1958, **68:**272–287.
98. Liu, C.: Studies on primary atypical pneumonia. I. Localization, isolation and cultivation of a virus in chick embryos. J. Exptl. Med., 1957, **106:**455–466.
99. Loosli, C. G., Hamre, D., and Gerber, P.: Antigenic variants of influenza A virus (PR8 strain). V. Virulence, antigenic potency, and cross-protection tests in mice of the original and second series. J. Exptl. Med., 1958, **107:** 857–868.
100. Marston, R. Q.: Cytopathogenic effects of hemadsorption virus type I. Proc. Soc. Exptl. Biol. Med., 1958, **98:**853–856.
101. Marston, R. Q., and Vaughan, E. R.: Parainfluenza 3—assay and growth in tissue culture. Proc. Soc. Exptl. Biol. Med., 1960, **104:**56–60.
102. McClelland, L., et al.: Studies of acute respiratory illnesses caused by respiratory syncytial virus. 2. Epidemiology and assessment of importance. New England J. Med., 1961, **264:**1169–1175.
103. McKinney, R. W., England, B. L., and Froede, S.: Studies with hemadsorption virus type 1. I. Recovery from two cases of influenza-like disease in military personnel and related investigations. Amer. J. Hyg., 1959, **70:**280–296.
104. Mims, C. A.: An analysis of the toxicity for mice of influenza virus. Brit. J. Exptl. Path., 1960, **41:**586–592; 593–598.
105. Mogabgab, W. J., and Holmes, B.: 2060 and JH viruses in secondary monkey kidney cultures. J. Infect. Dis., 1961, **108:**59–62.
106. Mogabgab, W. J., Holmes, B., Brightwell, R. J., and Resler, C. W.: Effect of dosage and interval between injections on the antibody response to polyvalent influenza vaccine. J. Lab. Clin. Med., 1961, **58:**391–404.
107. Mogabgab, W. J., Pelon, W., Burch, G. E., and Holmes, B.: Characteristics of the Asian strain of influenza A. Proc. Soc. Exptl. Biol. Med., 1958, **99:**116–120.
108. Morgan, C., Rose, H. M., and Moore, D. H.: Structure and development of viruses observed in the electron microscope. III. Influenza virus. J. Exptl. Med., 1956, **104:**171–182.
109. Morris, J. A., Blount, R. E., Jr., and Savage, R. E.: Recovery of cytopathogenic agent from chimpanzees with coryza. Proc. Soc. Exptl. Biol. Med., 1956, **92:**544–549.
110. Mulder, J.: Asiatic influenza in the Netherlands. Lancet, 1957, **ii:**334.
111. Mulder, J., and Masurel, N.: Pre-epidemic antibody against 1957 strain of Asiatic influenza in serum of older people living in the Netherlands. Lancet, 1958, **i:**810–814.
112. Neilson, D. B.: Sudden death due to fulminating influenza. Brit. Med. J., 1958, **i:**420–422.
113. Parker, W. L., Wilt, J. C., and Stackiw, W.: Adenovirus infections. Canadian J. Pub. Health, 1961, **52:**246–251.
114. Parrott, R. H., et al.: Clinical features of infection with hemadsorption viruses. New England J. Med., 1959, **260:**731–738.
115. Paul, J. H., and Freeze, H. L.: Epidemiological and bacteriological study of "common cold" in isolated Arctic community (Spitsbergen). Amer. J. Hyg., 1933, **17:**517–535.
116. Peacock, D. B., and Clarke, S. K. R.: Respiratory syncytial virus in Britain. Lancet, 1961, **ii:**466.
117. Pelon, W.: Classification of the "2060" virus as ECHO 28 and further studies of its properties. Amer. J. Hyg., 1961, **73:**36–54.
118. Pelon, W., and Mogabgab, W. J.: Further studies on 2060 virus. Proc. Soc. Exptl. Biol. Med., 1959, **102:**392–395.
119. Pelon, W., Mogabgab, W. J., Phillips, I. A. and Pierce, W. E.: A cytopathogenic agent isolated from Naval recruits with mild respiratory illnesses. Proc. Soc. Exptl. Biol. Med., 1957, **94:**262–267.
120. Pereira, H. G.: Typing of adenoidal-pharyngeal-conjunctival (APC) viruses by complement fixation. J. Path. Bact., 1956, **72:**105–109.
121. Pereira, M. S., and Fisher, O. D.: An outbreak of acute laryngotracheobronchitis associated with parainfluenza-2 virus. Lancet, 1960, **ii:**790–791.
122. Petersdorf, R. G., Fusco, J. J., Harter, D. H., and Albrink, W. S.: Pulmonary infections complicating Asian influenza. Arch. Intern. Med., 1959, **103:**262–272.
123. Petersen, K. B.: Studies on the properties of a new cytopathogenic and hemagglutinating virus (Copenhagen 222). Acta Path. Microbiol. Scand., 1959, **45:**213–224.
124. Petersen K. B., and von Magnus, P.: Isolation of a new virus from a child with influenza-like disease. Danish Med. Bull., 1958, **5:** 157–159.
125. Philipson, L., and Wesslen, T.: Recovery of a cytopathogenic agent from patients with nondiphtheritic croup and from day nursery children. Arch. f. d. gesamte Virusf., 1958, **8:**77–94; 192–203; 204–215; 332–350.
126. Price, W. H.: The isolation of a new virus associated with respiratory clinical disease in humans. Proc. Nat. Acad. Sci., 1956, **42:** 892–896.
127. Price, W. H., et al.: Studies of the JH and 2060 viruses and their relationship to mild upper respiratory disease in humans. Amer. J. Hyg., 1959, **69:**224–249.
128. Reichelderfer, T. E., et al.: Infection of human

volunteers with type 2 hemadsorption virus. Science, 1958, **128:**779–780.
129. Reilly, C. M., et al.: Studies of acute respiratory illnesses caused by respiratory syncytial viruses. 3. Clinical and laboratory findings. New England J. Med., 1961, **264:**1176–1182.
130. Report: Asian variant influenza type A. Pub. Health Repts., 1958, **73:**99–178.
131. Report: Expert Committee on Respiratory Virus Diseases. First report, Stockholm, 11–15 August 1958. World Health Organization, Palais des Nations, Geneva. 1959.
132. Roberts, G. B. S.: Fulminating influenza. Lancet, 1957, **ii:**944–945.
133. Rosen, L.: Hemagglutination-inhibition technique for typing adenoviruses. Amer. J. Hyg., 1960, **71:**120–128.
134. Rosen, L., Baron, S., and Bell, J. A.: Four newly recognized adenoviruses. Proc. Soc. Eptl. Biol. Med., 1961, **107:**434–437.
135. Rowe, W. P., et al.: Isolation of a cytopathogenic agent from human adenoids undergoing spontaneous degeneration in tissue culture. Proc. Soc. Exptl. Biol. Med., 1953, **84:** 570–573.
136. Rowe, W. P., Hartley, J. W., and Huebner, R. J.: Serotype composition of the adenovirus group. Proc. Soc. Exptl. Biol. Med., 1958, **97:**465–470.
137. Rowe, W. P., and Huebner, R. J.: Present knowledge of the clinical significance of the adenoidal-pharyngeal-conjunctival group of viruses. Amer. J. Trop. Med. Hyg., 1956, **5:**453–460.
138. Schaeffer, M., Robinson, R. Q., and Fodor, A. R.: New viruses and virus diseases of man. Ann. Rev. Microbiol., 1959, **13:**335–348.
139. Schultz, E. W., and Habel, K.: SA virus—a new member of the myxovirus group. J. Immunol., 1959, **82:**274–278.
140. Sellers, T. J., Jr., et al.: The influence of influenza virus on exogenous staphylococcal and endogenous murine bacterial infection of the broncho-pulmonary tissues of mice. J. Eptl. Med., 1961, **114:**237–256.
141. Shelokov, A., Vogel, J. E., and Chi, L.: Hemadsorption (adsorption-hemagglutination) test for viral agents in tissue culture with special reference to influenza. Proc. Soc. Exptl. Biol. Med., 1958, **97:**802–809.
142. Shope, R. E.: The swine lungworm as a reservoir and intermediate host for swine influenza virus. J. Exptl. Med., 1943, **77:**127–138.
143. Shope, R. E.: The swine lungworm as a reservoir and intermediate host for swine influenza virus. V. Provocation of swine influenza by exposure of prepared swine to adverse weather. J. Exptl. Med., 1955, **102:** 567–572.
144. Shope, R. E.: Influenza history, epidemiology and speculation. Pub. Health Repts., 1958, **73:**165–178.
145. Siegert, R., Enders, G., and Hecker, A.: Parainfluenza-Infektionen in Westdeutschland. Deut. med. Woch., 1961, **86:**1893–1899; 1939.
146. Siegert, R., Falke, D., Dietrich, F., and Friolet, B.: Influenzavirus-Infektionen des Typ D/ Sendai in Deutschland. Deut. med. Woch., 1959, **84:**659–663.
147. Sommerville, R. G.: Epidemic kerato-conjunctivitis—an adenovirus infection. J. Hyg., 1958, **56:**101–107.
148. Sugg, J. Y., and Cleeland, R.: Differences in trypsin susceptibility among influenza viruses and relationship of the susceptibility to the antigenic type or subtype of the virus. J. Immunol., 1962, **88:**369–376.
149. Sutton, R. N. P., Clarke, S. K. R., and Tyrrell, D. A. J.: Newly recognized myxoviruses in Europe. Lancet, 1959, **i:**395.
150. Symposium: Viruses in search of disease. Part II. New respiratory and ocular viruses. Ann. N. Y. Acad. Sci., 1957, **67:**255–303.
151. Symposium: Discussion on adenovirus infections. Proc. Roy. Soc. Med., 1957, **50:**753–760.
152. Symposium: Discussion on virus infections of the upper respiratory tract. Proc. Roy. Soc. Med., 1958, **51:**469–474.
153. Symposium: The cytopathology of virus infection. Ann. N. Y. Acad. Sci., 1959, **81:**1–214.
154. Symposium: Respiratory syncytial virus. J. Amer. Med. Assn., 1961, **176:**647–667.
155. Tyrrell, D. A. J., and Bynoe, M. L.: Inoculation of volunteers with JH strain of new respiratory virus. Lancet, 1958, **ii:**931–933.
156. Tyrrell, D. A. J., and Bynoe, M. L.: Some further virus isolations from common colds. Brit. Med. J., 1961, **i:**363–372.
157. Tyrrell, D. A. J., et al.: Inoculation of human volunteers with parainfluenza viruses types 1 and 3 (HA 2 and HA 1). Brit. Med. J., 1959, **ii:**909–911.
158. Tyrrell, D. A. J., et al.: Some virus isolations from common colds. I. Experiments employing human volunteers. Lancet, 1960, **i:**235–237.
159. Tyrrell, D. A. J., and Parsons, R.: Some virus isolations from common colds. III. Cytopathic effects in tissue cultures. Lancet, 1960, **i:**239–242.
160. Utz, J. P., et al.: Clinical and laboratory studies of mumps. I. Laboratory diagnosis by tissue-culture technics. New England Med. J., 1957, **257:**497–502.
161. Utz, J. P., Szwed, C. F., and Kasel, J. A.: Clinical and laboratory studies of mumps. II. Detection and duration of excretion of virus in urine. Proc. Soc. Exptl. Biol. Med., 1958, **99:**259–261.
162. Valentine, R. C., and Isaacs, A.: The structure of the Newcastle disease-mumps-influenza (myxovirus) group. J. Gen. Microbiol., 1957, **16:**680–685.
163. Vargosko, A. J., et al.: Association of type 2 hemadsorption (parainfluenza 1) virus and Asian influenza A virus with infectious croup. New England Med. J., 1959, **261:** 1–9.
164. Ward, T. G.: Viruses of the respiratory tract. Progress Med. Virol., 1959, **2:**203–234.
165. Zhdanoff, V. M., Ritova, V. V., and Golygina, L. A.: Influenza D in early infancy. Acta Virol., 1957, **1:**216–219.
166. Zuschek, F.: Studies of the hemagglutinins of types 3, 4 and 7 adenovirus. Proc. Soc. Exptl. Biol. Med., 1961, **107:**27–30.

Chapter 39

THE ENTERIC VIRUSES: POLIOMYELITIS, VIRUSES, COXSACKIE VIRUSES, ECHO VIRUSES; THE HEPATITIS VIRUSES

THERE ARE a number of viruses infecting man which are found in the bowel early in the infection, even though the predominant symptoms of the disease are referable to effects on other tissues, such as of the central nervous system, infected as a consequence of hematogenous spread of the virus. These include viruses such as the poliomyelitis viruses and the viruses of mouse encephalitis and of Teschen disease of swine, the Coxsackie viruses, and the so-called orphan viruses of man and lower animals, and these are grouped together as the enteric viruses or enteroviruses.[96, 117, 144] These viruses are morphologically similar in that they are spherical in shape and are among the smallest of the viruses, 15 to 35 mμ in diameter, and are cultivable in a variety of tissue cell cultures.[76] The diseases they produce have common epidemiological features. It is not clear to what extent the viruses of infectious and serum hepatitis are related to the above viruses. In infectious hepatitis the virus is present in the feces early in the disease, or even during the incubation period, suggesting the possibility of an alimentary infection, but the virus of serum hepatitis is apparently present only in the blood, and seems not to occur in the bowel.

POLIOMYELITIS VIRUSES[32, 95]

Poliomyelitis (infantile paralysis, acute anterior poliomyelitis, Heine-Medin disease) was differentiated as a clinical entity by Heine in 1840, and described in epidemic form by Medin in 1891. The viral nature of the etiological agent was demonstrated in 1908 when Landsteiner and Popper transmitted the disease to monkeys.

The infection is widely distributed thoughout most of the world, and the occurrence of the disease in young children for the most part is, as in many other diseases of childhood, an expression of a high probability of contact with the polioviruses. It is estimated that in Chicago most children have been infected once within the first 4 years of

life, and by the sixth birthday 75 per cent have been infected twice.[18] The age at which primary infection occurs is subject to some variation and is affected by social and economic factors (see below).

The great majority of infections are symptomless, and the disease, paralytic or nonparalytic, appears to be the exception. With the waning of passive immunity of maternal origin, repeated infection, with or without disease, results in an immunity, and most adult serums contain neutralizing antibody which is invariably present in pooled materials such as γ-globulin concentrates. The incidence of the disease is similar to that in certain other infections, such as meningococcal infection, in which the causative agent is widely distributed in the human population, but the infection is usually symptomless, and the disease tends to appear in sporadic form or occasionally in epidemics.

Poliovirus.[140] These are among the smallest of the viruses and estimations of size of the spherical particle, based on differential filtration, sedimentation, and direct observation in electron micrographs, range from 22 to 32 mμ in diameter. The virus particles may occur in orderly arrays and have been prepared in the form of bipyrimidal crystals (Chap. 3). Analysis of highly purified preparations has given a nitrogen-phosphorus ratio of 12.4 in the case of type 2 virus,[143] and the virus substance appears to be largely nucleoprotein. Ribonucleic acid makes up about 24 per cent of the particle, and DNA, if present, accounts for less than 1 per cent of the total.

The activity of the virus is stable over a relatively wide pH range, pH 4 to pH 10, over periods of several days at refrigerator temperature; it is resistant to treatment with glycerol, ether, and 1 per cent phenol but is labile to oxidizing agents and ultraviolet irradiation. It is inactivated in 30 minutes at 50 to 55° C. and destroyed in milk by pasteurization. It persists in feces and sewage at refrigerator temperatures for extended periods, for as long as 3 months, and in water has been reported to be somewhat more resistant to chlorination than the vegetative cells of many bacteria. Its inactivation by formalin has been of interest since this substance is used in the preparation of inactivated vaccines, and prolonged treatment results in the disappearance of antigenicity. The rate of inactivation is exponential over a considerable range (Chap. 6), but treatment based on extrapolation of this rate has resulted in incomplete inactivation.[21, 44, 139]

Antigenic types. Strains of poliovirus are not necessarily immunologically identical, and these viruses have been separated into three types by the specificity of neutralizing antibody. These types are numbered, and are also designated by the name of the prototype strain, *e.g.*, type 1 or Brunhilde, type 2 or Lansing, and type 3 or Leon. Minor antigenic differences occur between strains of all three types.[33, 155, 156] Of these, type 1 is found most often, but more than one type may occur in a single epidemic. Typing is carried out by cross-neutralization tests, at first with monkeys, but now largely in tissue culture. Of a group of 100 strains of global origin typed in the monkey, eighty-five were found to be type 1, twelve type 2, and three type 3; of a group of 274 strains isolated in this country and typed in tissue culture, 204 were type 1, twenty-two were type 2, and forty-eight were type 3. The distribution of the types appears to be world-wide, but it is uncertain as to whether figures such as these reflect the relative prevalence of the three types in the human population.[145] These types differ somewhat in other ways. For example, the Lansing strain is one of the more readily adaptable to rodents, and the Leon type has relatively low virulence for the monkey. There are also differences in virulence between strains, the Brunhilde strain being one of the more virulent strains of type 1.

Two kinds of complement-fixing antigen are present in the virus which are separable by density gradient centrifugation, fraction C and fraction D, with infectivity associated largely with the

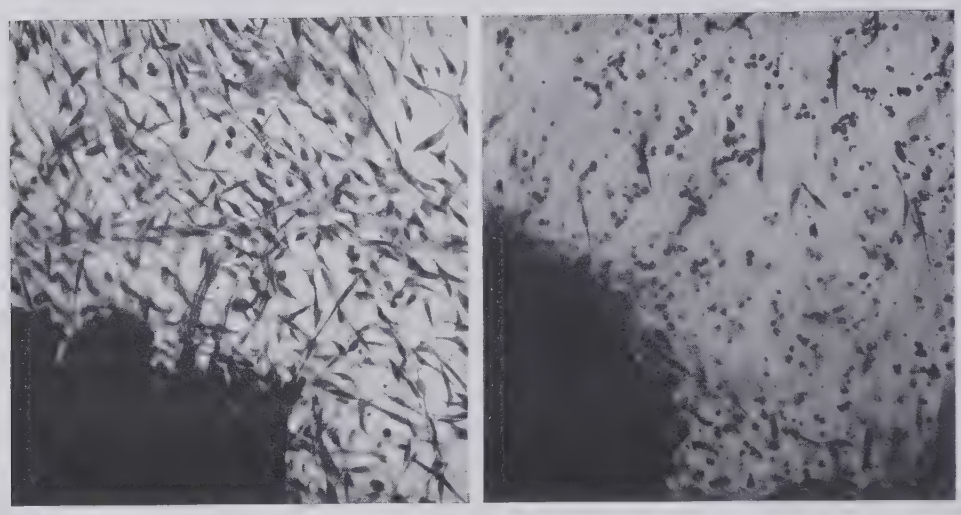


Figure 287. Preparations from roller-tube cultures of human embryonic skin-muscle tissue 7 days after cultures were initiated. *Left,* uninoculated culture; *right,* inoculated 3 days previously with the Brunhilde strain of poliomyelitis virus. Few cells remain in the infected culture, the majority having been destroyed by the cytopathogenic action of the virus. Hematoxylin and eosin stain. (Weller, New England J. Med.)

latter, heavier, fraction.[90, 123] These antigens are separable by gel-diffusion,[85] and D is heat-labile while C increases in amount on heating, presumably as a result of a D → C conversion rather than an unmasking of C.[86] The occurrence of these two kinds of antigens resolves to a considerable extent anomalous results previously obtained with the complement-fixation reaction. Some workers have introduced the designation N for infectious antigen and H for heat-stable antigen, *i.e.,* C = H and D = N, since it is not established that all particles which are antigenically C invariably sediment in the C zone characteristic of empty shells.[70, 71]

Tissue culture. Beginning in 1949, poliovirus has been grown in cultures of a variety of tissues, including human embryonic tissues, human foreskin and testis, monkey kidney and testis, and in HeLa cell culture, in the Maitland-type flask culture, and in roller and static tube culture. By appropriate adjustment and timing of inoculums, interference (Chap. 4) among the three immunological types is demonstrable.

A degenerative type of cytopathology is produced which is clearly apparent in the fibroblastic outgrowth as a progressive granulation and eventual destruction.[63] The cytopathogenic effect is more pronounced with some virus strains than others, *e.g.,* the Lansing strain is feebly cytopathogenic while the Y-SK strain, also type 2, produces extensive degeneration. Further, pathogenicity in tissue culture and pathogenicity for experimental animals are not necessarily parallel.

The cytopathogenic effect is specifically neutralized by antiserum, thus providing the basis for tissue culture typing of virus strains and may be quantitated for virus assay by the plaque method.[26] There is evidence that the observed cytopathology is in part a toxic effect of some strains of poliovirus in which the toxicity and infectivity are immunologically independent.[1]

Poliovirus may also be grown in tissue culture by the monolayer technique in which the layer of cells is inoculated, incubated for 45 minutes, and, after the excess inoculum is removed, is overlaid with agar. Discrete foci of degeneration, or plaques, occur when the inoculum is sufficiently dilute and may yield differentiable virus strains on subculture.

Variation. Serial passage in tissue culture may result in changes in virulence for experimental animals. The Lansing strain, carried in human embry-

onic tissue culture, for example, showed a decrease in mouse virulence without a corresponding decrease in virulence for the monkey or alteration in tissue culture infectivity, and the Brunhilde strain, carried similarly, showed a marked decrease in virulence for the monkey.[40] In the same way, after passage in monkey testis tissue culture, the Lansing strain lost its pathogenicity for the mouse but retained its virulence for the monkey,[87] but adaptation to rodent hosts is associated with loss of virulence for the monkey.[79]

Variation in virulence for the mammalian host may be accompanied by changes in plaque morphology, as in the case of the *d* variant.[34, 152] Variants of reduced virulence but with, or perhaps because of, abnormal capacity for growth in tissue culture have been produced by rapid tissue passage with large inoculums by Sabin and his associates.[135] These have been of special interest as immunizing agents. (see below)

Disease in Man. So far as is known, natural infection with poliovirus occurs only in man. The virus is demonstrable in the pharyngeal secretions and stools of infected individuals, including contacts who do not develop the disease, and there appears to be a large reservoir of infection in the human population. The infection is spread by contact, though in precisely what way is uncertain, and cases of the disease are distributed radially from an original focus of infection. The incubation period is variable, and is usually given as 1 to 2 weeks, although it may be as long as 4 or 5 weeks. The clinical disease is separable into three types, the abortive, the nonparalytic, and the paralytic.

The initial symptoms of the disease are those of a mild upper respiratory infection with nonexudative pharyngitis and headache, or of gastroenteritis with nausea and vomiting, but constipation rather than diarrhea, and in either case there is a febrile reaction. When the disease does not develop beyond this point, it is known as abortive poliomyelitis, but it cannot be diagnosed as such without isolation of the virus or demonstration of an increase in antibody titer between paired serums.

The disease may progress with the development of symptoms of involvement of the central nervous system, including pain and stiffness of the muscles of the neck and back and Kernig's sign. This is nonparalytic poliomyelitis. In paralytic poliomyelitis a flaccid paralysis and spasm of unparalyzed or partially paralyzed muscles occur at the terminal phase of the febrile period, progressing for only a short time, usually not beyond 24 hours. Virus disappears rapidly from the spinal cord but may continue to be eliminated in the feces for some time.

The lesions in paralytic poliomyelitis consist of pathological changes in neu-

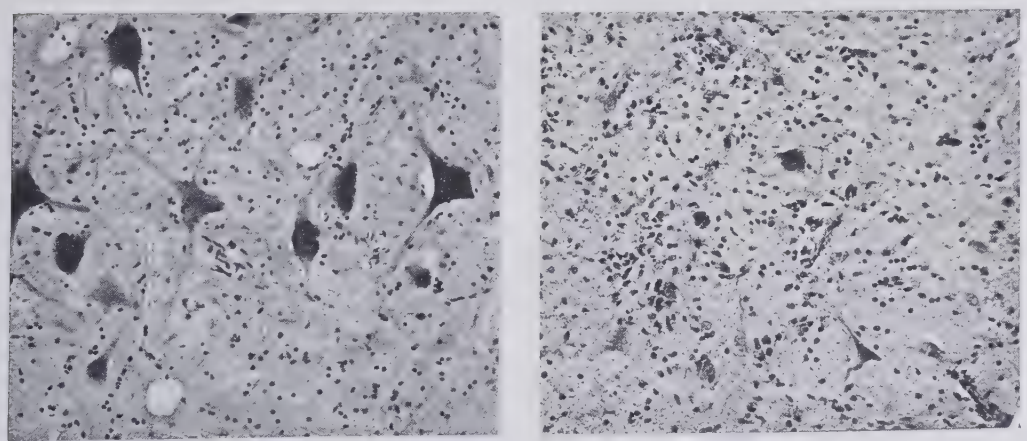


Figure 288. Sections through the anterior horn of monkey spinal cords. *Left,* normal monkey; *right,* monkey paralyzed with poliomyelitis showing the destruction of the large anterior horn cells and neuronophagia. Hematoxylin and eosin; × 125.

rons, ranging from mild chromatolysis to complete destruction, and are characterized by local perivascular infiltration with lymphocytes and macrophages, with phagocytosis of the necrotic motor cells. The anterior horn of the spinal cord is involved, and the destruction of the large nerve cells giving rise to the motor fibers of the peripheral nerves results in the flaccid paralysis. The selective distribution of lesions in the brain is distinctive, differentiating poliomyelitis from the viral encephalitides in which there is a more or less general diffusion of the virus into the brain. In poliomyelitis the lesions in the cerebral cortex are confined to the motor and premotor areas, rarely extending into the postcentral cortex, and the visual, auditory, and association areas are not involved. There is no impairment of mental faculties, and symptoms of encephalitis disappear when the acute stage of the disease subsides. In the hindbrain the motor cells of the medulla and pons, the vestibular nuclei, and related centers in the cerebellum may be involved, but the neocerebellar complex is not affected. Bulbar poliomyelitis is a consequence of a concentration of neuron destruction in the parts of the medulla containing the motor nuclei of the cranial nerves, with effects ranging from weakness of facial muscles to involvement of the respiratory and vasomotor centers.

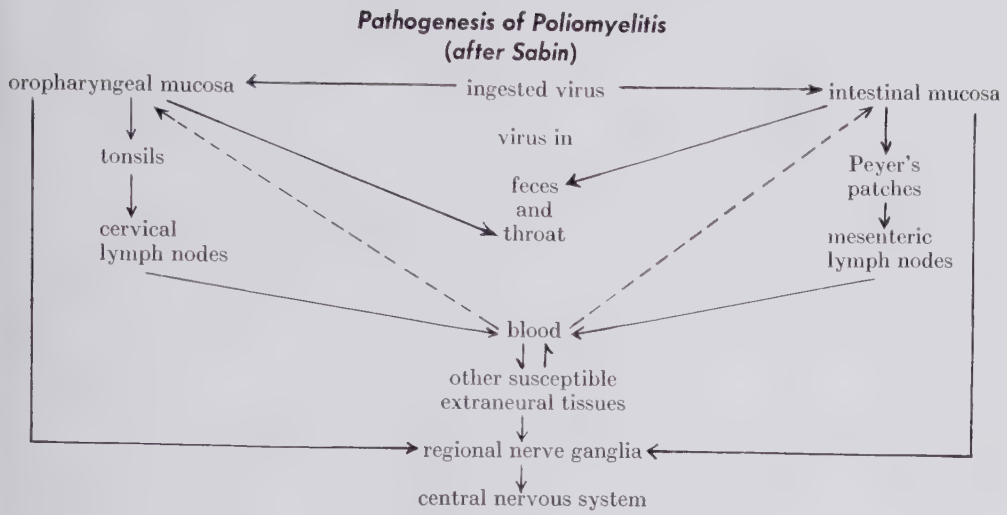
Pathogenesis. The pathogenesis of poliomyelitis has been worked out in detail in the champanzee and, since this experimental infection appears to be a counterpart of human infection, may be assumed to represent, in a general way at least, the pathogenesis of the human disease. According to Bodian,[10, 11] within a few days after the ingestion of an infecting dose high concentrations of virus are demonstrable in the lymphoid tissue in the tonsils and in Peyer's patches in the intestine. Multiplication of the virus and its excretion from these areas account for its appearance in throat swabs and washings and in the feces. The deeper lymph nodes, cervical and mesenteric, are infected by drainage from these primary sites, and the virus invades the blood stream by way of the lymphatic vessels and the thoracic duct to produce viremia. The viremia is seldom demonstrable in man, possibly because it occurs very early. The infection is spread via the blood stream to various susceptible tissues, including other lymphatic tissue, brown fat, and the central nervous system.

The pathway of invasion of the central nervous system is somewhat uncertain. It may be by way of the blood, but there is also evidence of centripetal spread of the virus along the axons of peripheral nerves, and Sabin[129] regards the regional nerve ganglia and their connections with the central nervous system as playing a significant role in the pathogenesis of the disease. Virus and lesions are not present in the olfactory bulbs, and virus occurs only rarely in human celiac ganglia, but the vagus and visceral afferent fibers represent a possible pathway from the gastrointestinal tract to the central nervous system. It is also possible that lymphatic tissue is not the exclusive site of multiplication of the virus, but other extraneural tissues may also serve as foci of infection.

The virus is present in the feces and laboratory diagnosis is dependent upon its isolation and serological identification. Monkey kidney, HeLa, KB and human amnion cell cultures appear to be about equally sensitive for isolation.[19] Serological identification consists of neutralization in tissue culture with known antiserum[110] and the fluorescent antibody technique has been applied also.[54]

Predisposing factors. In the abortive type of disease, the infection progresses no further than the alimentary or viremic phase, a limited invasion of the central nervous system with reparable damage occurs in nonparalytic poliomyelitis, and extensive invasion and destruction within the central nervous system, which may be largely spinal, or bulbar and bulbospinal, occurs in the paralytic form. The factors which determine whether the virus is to enter and multiply within the central nervous system are known in only a limited way.

There is no doubt that specific immu-



nity plays an important part in limiting spread of the infection, but certain nonspecific factors are significant also. Evidence from various sources indicates that injections for immunization of children against diphtheria, pertussis, and tetanus may influence to some degree the site of paralysis in cases occurring up to approximately 3 weeks following the inoculation, but it is not clear that the incidence of paralysis is affected.[50, 80] It is also established that heavy physical exercise during the prodromal stage of the disease results in more severe paralysis, and paralysis is more likely to occur during pregnancy. An increased incidence of the bulbar form of the disease occurs following tonsillectomy or adenoidectomy. The surgical trauma may open the way for virus already present in the pharnyx to gain access to the cranial nerves by which it may reach the medulla directly, or local changes may occur in the permeability of the vascular system supplying the neuraxis.[9, 104] In any case, elective operations in the oropharyngeal area, including dental extractions, should not be performed during epidemics of poliomyelitis, and preferably not during the late summer and early fall when the prevalence of the infection is high.

There is also no doubt that age is highly significant in the severity of the disease. It is less severe in young children than adults as indicated by an increase in both disability and case fatality with increasing age. The incidence of the more severe bulbar type of infection is appreciably greater in the higher age groups, and the case fatality rate is two to five times as great in adults as in children under 5 to 7 years of age.

Epidemiology. The influence of social and economic conditions on the epidemiological character of infectious disease has been discussed elsewhere (Chap. 10) and is nowhere better illustrated than in the changing epidemiological character of poliomyelitis. As indicated above, the virus of poliomyelitis is widely disseminated in the human population, the vast majority of infections are asymptomatic or abortive, and the paralytic disease is a relatively rare occurrence.

The epidemic described by Medin in 1891 in Sweden is regarded as one of the first epidemics to occur, and the first major epidemic in this country occurred in 1916. Beginning about the turn of the century the disease began to shift from an endemic form, characterized by sporadic cases, to a form in which cases occur in epidemics. The endemic form continues to persist in primitive populations, particularly in tropical areas,[2, 107] but epidemics have begun to occur in parts of Africa and India, and Japan appears to be in transition from the endemic to the epidemic form of the disease. Coincident with this shift in

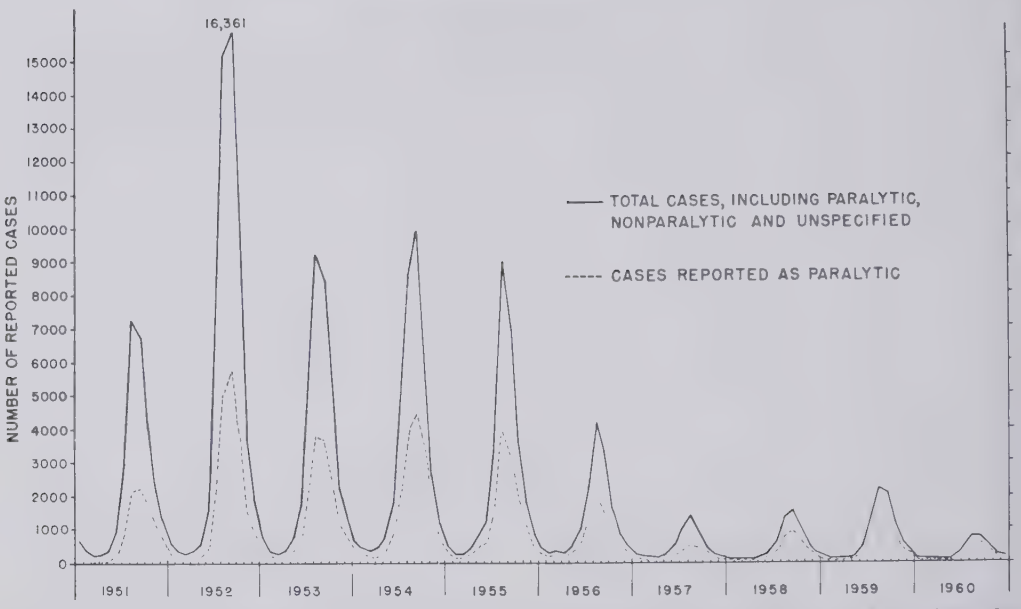


Figure 289. Reported cases of poliomyelitis in the United States, 1951–1960. Reproduced from the Morbidity and Mortality Weekly Report Annual Supplement, Vol. 9, No. 53. Communicable Disease Center, Public Health Service, U. S. Department of Health, Education and Welfare.

the character of the disease, the peak in age incidence has shifted from the 0 to 4 age group to the 5 to 9 age group, and increasing numbers of cases occur in higher age groups and in adults.

This change in epidemiological character appears to be a consequence of the operation of social and economic factors which postpone the time of primary exposure to the virus.[47, 97] Under relatively primitive conditions, transfer of infection occurs more readily than under more enlightened hygienic circumstances. Primary infection may well occur early enough that passive immunity of maternal origin modifies, in the aggregate, the early immunizing infections, with the result that a pseudo-racial immunity develops, and the population is immune to epidemic disease without experiencing clinical disease in epidemic form. By postponement of the first infection, however, the threshold density of susceptibles is raised to a point at which the population becomes susceptible to epidemic disease.

It is sometimes possible to interfere successfully with the development of population susceptibility to epidemic disease by prophylactic immunization. In diphtheria, for example, while the proportion of susceptibles constituting the threshold density is not known with precision, prophylactic immunization of 70 per cent of school age children suffices to prevent epidemics of that disease. Similarly, in poliomyelitis, the development of effective immunizing agents and their application on a sufficiently wide scale may also result in the reversion of this disease to the endemic form characterized by sporadic cases. A trend in this direction, following the introduction of immunization on a wide scale in 1954, is suggested by a rise in the relative incidence of the disease in the 0 to 4 age group in 1956. Other factors may be operative also; there is, for example, evidence[29] of a similar trend in some areas in 1948 prior to immunization which may be due to factors such as changing birth rates and the incidence of disease in prior years.

The seasonal incidence of the disease, characterized by a peak in late summer and early fall, was for a time thought to suggest an insect vector. Although virus may persist in muscoid flies under experimental conditions and virus has been isolated from flies having access to

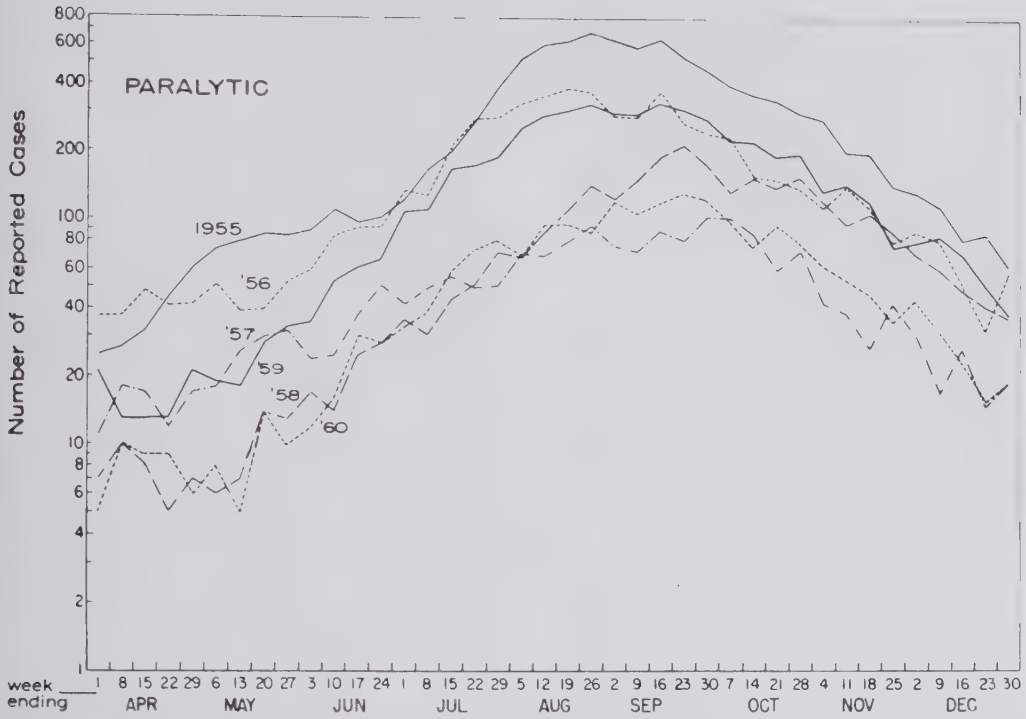


Figure 290. The seasonal incidence of poliomyelitis in the United States as illustrated by the reported cases of paralytic poliomyelitis for the years 1955–1960 inclusive. Note that the "poliomyelitis year" extends from April through December. Reproduced from the Morbidity and Mortality Weekly Report Annual Supplement, Vol. 9, No. 53. Communicable Disease Center, Public Health Service, U. S. Department of Health, Education and Welfare.

human feces during an epidemic, fly control programs have not affected the incidence of the disease, and other epidemiological evidence does not indicate that the infection is transmitted by an insect vector.

Experimental Infections. For many years it was believed that only primates could be experimentally infected with poliovirus. Of such animals available for experimental purposes, the chimpanzee is the most susceptible and has, in fact, been infected accidentally in the laboratory, and the disease in this animal resembles the human disease closely. Of the commonly used monkeys, the cynomologous monkey may be infected by the gastro-intestinal route, and the rhesus is more resistant but may be infected by intracerebral inoculation. The findings at autopsy are similar to those observed in man, with pathological changes, including neuron destruction and perivascular infiltration in the gray matter of the cord.

In 1939 the Lansing strain of virus was adapted from the monkey to the cotton rat and then to mice, and later strains of the other two immunological types have been adapted to mice by introduction of the virus directly into the spinal cord. In such rodents the incubation period is 2 to 10 days, there is paralysis of one or more legs, few recover, and death occurs from respiratory paralysis. Type 2 strains will also infect hamsters, and one such strain, after many passages in suckling hamsters, has been adapted to the chick embryo.[122]

Immunity. The immune response to poliovirus is demonstrable as the presence of complement-fixing and neutralizing antibodies in the serum and a resistance to second inoculation. Serum antibody fixes complement in the presence of tissue culture antigen, but its specificity may overlap the serotypes of the virus, possibly in part because of an anamnestic heterotypic antibody response. Complement-fixing antibody

declines relatively rapidly, disappearing in from within a few months to 2 years. Neutralizing antibody may be titrated in the monkey, but much more readily in tissue culture. Neutralization of the virus is indicated by the lack of development of cytopathology in the tissue culture or by changes in pH shown by the inclusion of phenol red in the tissue culture fluid. In contrast to complement-fixing antibody, it persists in detectable titer for years. Both kinds of antibody appear quite early in the disease, but if acute phase serum is taken early enough, a rise in antibody titer may be shown to occur between this serum and a convalescent serum taken 3 to 4 weeks after onset of the disease.

The presence of neutralizing serum antibody is associated with an effective immunity to the disease. As indicated above, the proportion of individuals showing neutralizing antibody increases sharply with age, and the great majority of adult serums are found to contain antibody. At one time this was thought to represent a "maturation" immunity but, as in many other infectious diseases of childhood, it is now clear that the immunity is a specific response to repeated symptomless or abortive infections which need not progress beyond the alimentary, or possibly viremic, stages to induce the immune response.

Although second, and even third, attacks of poliomyelitis have been found to occur in the same individual, it is probable, although difficult to prove in man, that the second attack is caused by an immunological type of virus different from that of the prior infection. Monkeys recovered from one attack of the disease are solidly immune to inoculation with the same type of virus, but not to heterologous types, and it is possible that this is true in man also. Naturally acquired active immunity in man is probably an immunity to all three types of poliovirus, continuously reinforced by repeated exposure and possibly symptomless infection.

Prophylaxis. Artificially acquired prophylactic immunity may be either passive or active in nature. The source of antibody in the first instance is the concentrated antibody present in pooled γ-globulin. Passive immunization has been shown to be effective in experimental animals and in man has been found to reduce the number of cases of paralytic disease by more than half, providing that the globulin is administered within the first few days of the incubation period,[52] but serum therapy of the established disease is ineffective. Passive immunity disappears rapidly, and is no longer effective after about 3 weeks.

Active immunization. Active immunity may be produced by parenteral inoculation with inactivated vaccine prepared from monkey kidney cell culture, or by infection with attenuated strains of the virus by the oral route. Both kinds of vaccines must include all three immunological types of the virus to provide broad protection.

Inactivated vaccine. Vaccines inactivated with formaldehyde were prepared by Salk[136] and are known as Salk vaccine. This kind of vaccine was subjected to extensive field tests, beginning in 1954, and was shown to provide an effective immunity as indicated by a reduction of 70 to 80 per cent in the incidence of the paralytic disease when given as a course of three inoculations[42, 82, 83, 118, 148] and there is evidence that the disease is less severe in inoculated persons,[114] but the immunity is not completely effective.[8] A purified vaccine has been prepared[22] but its relative efficacy has not been determined. The preparation of an inactivated, yet immunologically potent, vaccine is technically complex,[92] owing to the effect of formaldehyde on antigenicity. The immunity so produced is assayed as protective serum antibody and is presumably dependent upon the presence of such antibody, *i.e.*, such immunization does not prevent the alimentary infection[41, 88] but is effective in the viremic stage and on hematogenous spread of the infection.

Living attenuated vaccine. Virus strains attenuated with respect to neurovirulence have been developed by various workers, largely Sabin, Koprowski,

and the Cox-Lederle group, for use as immunizing agents. The stability of such attenuated strains with respect to virulence and associated characteristics has been subjected to intensive study[149] and the probability of reversion to neurovirulence appears to be very small. Vaccines prepared from such strains[15] may be mono- or divalent and given separately to avoid overgrowth and/or interference effects, or in trivalent form, but only a single dose of a given preparation is required.

These vaccines have been tested in local areas in this country[28, 105, 134] where evaluation of large scale testing is complicated by prior Salk vaccine immunization, and in England,[119] and field trial has been carried out on a large scale elsewhere, especially in the Soviet Union.[24, 146, 147] The results of such testing have been excellent[16, 27, 108, 109, 130, 131] and the future of such vaccines as effective immunizing agents is very promising.

In addition to the accentuated antigenic stimulus provided by proliferation of the vaccine strains *in vivo* to give an alimentary infection and the consequent requirement for a single oral, rather than multiple parenteral, inoculation, such immunization differs from that produced by inactivated virus in two significant respects. First, immunization is against the alimentary infection and the immunity may be tested by challenge inoculation with attenuated virus. This kind of immunity tends to depress, if not effectively prevent, dissemination of virulent virus. Second, infection with attenuated virus tends to spread in the community to immunize or reinforce the immunity of others[60] and there is evidence suggesting that attenuated viruses may displace virulent virus in the intestinal tract. Such a combination of effects is perhaps the basis of the efficacy of oral attenuated vaccine in arresting poliomyelitis in West Berlin at the beginning of an epidemic prevalence.[78] The vaccine also produces an effective immunity in the very young; 75 per cent in newborn, and 95 per cent in infants 3 to 6 months of age has been described.[58, 106] Considerations such as these have led Sabin[133] to suggest that poliomyelitis may be eradicated in effectively immunized areas.

ENCEPHALOMYELITIS OF MICE

A spontaneous encephalomyelitis of mice of viral etiology was described, by Theiler in this country, by Gildemeister and Ahlfeld in Germany, and by Iguchi in Japan, in the 1930's, which is characterized by a flaccid, poliomyelitis-like paralysis. The infection is widespread in laboratory mice. The virus is present in the intestinal tract and mesenteric lymph nodes of most mice 4 to 6 weeks of age and is shed in the feces, but can no longer be found at about 6 months, and the mice are immune. The disease can be transmitted to uninfected mice by intracerebral, intranasal, or intraperitoneal inoculation. Following intracerebral inoculation, a flaccid paralysis develops after an incubation period of 5 to 30 days, and the histopathology includes perivascular infiltration in the central nervous system and necrosis of the ganglion cells of the anterior horn followed by neuronophagia. Rabbits and monkeys are not susceptible, and symptomless infection is produced in suckling hamsters following intracerebral inoculation.

The causative virus is immunologically distinct from the human polioviruses, but it resembles them, not only in the disease produced, but also in its small size, 25 to 30 mμ, and in its resistence to ether and glycerol.[93] The original virus found by Theiler is called Theiler's virus, or TO virus. Two other strains found later, FA and GD7 strains, appear to be less widely distributed. So far as is known, these viruses are nonpathogenic for man. They do constitute an artifact in the use of mice for virus studies; for example, the rodent-adapted strain of Lansing poliovirus became contaminated with FA virus in the

mouse and was purified by passage in the monkey which is not susceptible to FA virus infection.

TESCHEN VIRUS (PORCINE ENCEPHALOMYELITIS)[20]

A highly fatal disease of swine, characterized by a flaccid, ascending paralysis, was first described in the Teschen district of Czechoslovakia as Teschen disease, and spread over Europe under various names such as Talfan disease in England and poliomyelitis suum in Denmark. It is not known to occur in this country. The causative virus is smaller than the polioviruses, 10 to 15 mμ in diameter, and is immunologically unrelated to the polioviruses and also to many other neurotropic viruses infecting man. Apparently only the pig is susceptible to infection, and the disease is a diffuse encephalomyelitis affecting the cerebral cortex, the cerebellum, thalamus, basal ganglia, and anterior horn cells of the spinal cord in sharp distinction to the selective localization of the lesions produced by poliovirus.

COXSACKIE VIRUSES[67, 77, 94]

The first of what proved to be a new group of viruses was isolated by Dalldorf and Sickles in 1948 from two children, ill with disease diagnosed as poliomyelitis, by the inoculation of infant mice. Ten additional strains were isolated by Dalldorf the following year, and it was evident that some of the isolates differed from one another. Similar viruses were subsequently isolated by many investigators in various parts of the world. These viruses make up the somewhat heterogeneous group known as the Coxsackie viruses,* or C viruses.

These viruses have been found most often in feces and in sewage and are isolated from time to time from the throat.[45] They have been found together with poliovirus and during poliomyelitis epidemics in persons not infected with poliovirus. They appear to be most prevalent in the late summer and early fall; human infections seem to be quite common, as indicated by the presence of antibody to all of the immunological types of these viruses in pooled antibody such as γ-globulin; and they are found in man both in association with disease and in healthy persons.

Although of similar size, 15 to 35 mμ, the Coxsackie viruses are distinct from the polioviruses in pathogenicity and immunological character. They are relatively stable over a wide range of temperature and pH; preparations at near-neutrality are still active after long periods at room temperature, and for at least 1 day at pH extremes of 2.3 and 9.4. They are relatively resistant to substances such as ethyl alcohol, ether, glycerol, and phenolic compounds, but 0.3 per cent formaldehyde is an effective disinfectant.

Infection of cynomologous monkeys and chimpanzees by the oral route results in a transient febrile reaction, virus is found in the pharyngeal secretions and stools, there is a specific immune response, and some strains may produce neuron damage on intracerebral inoculation. Of the more common experimental animals, only the newborn mouse and hamster are susceptible to infection, although some strains occasionally infect adult mice to produce pancreatitis. While this limited host range is a definitive characteristic of the Coxsackie viruses, it does not differentiate them from certain other viruses, the Sinbis and dengue viruses (Chap. 40), and certain tissue culture–passed strains of ECHO viruses (see below).

Virus Types. These viruses are sepa-

* The first strains were found in Coxsackie, New York.

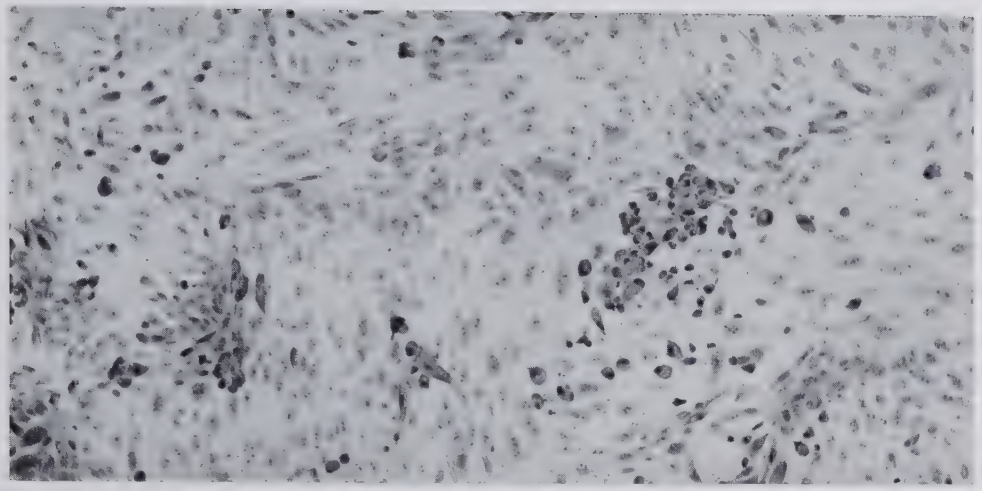


Figure 291. Coxsackie virus type B2 in monkey kidney epithelial cell culture. There are focal areas of cytopathology produced 24 hours after inoculation of the virus; the affected cells are rounded, contain crescentia, pyknotic nuclei, and the cytoplasm shows intense eosinophilic staining. (Enders, Ann. Intern. Med.)

rable into two groups, designated A and B, on the basis of their pathogenicity for the infant mouse and ability to grow in tissue culture, and into immunological types within these groups. The characteristic effect of the viruses of group A in the infant mouse is an inflammatory and degenerative lesion of striated muscle without adverse effect on other tissues. Infected mice show a generalized weakness, a flaccid paralysis develops, and death occurs 3 to 4 days after inoculation. At autopsy the skeletal muscles appear white and firm, and a hyaline degeneration is found on microscopic examination.

In contrast, infection with group B viruses characteristically results in lesions of the central nervous system, with focal leptomeningitis and perivascular lymphocytic infiltration, necrosis of the acinar tissue of the pancreas, hepatitis, and a necrotizing steatitis of the interscapular, cervical, and cephalic pads, and some strains produce focal lesions in the muscles, especially after intraperitoneal inoculation. The occurrence or predominance of these kinds of lesions is variable from one strain of virus to another and is also affected by the age of the mice. Irregular tonic contractions of the muscles prior to death may result in symptoms of "rolling disease" similar to that produced by some of the pleuropneumonia-like organisms (Chap. 27). Passage in the infant mouse may be required before the pathological picture becomes characteristic, and the distinction between the A and B groups is not necessarily sharp because of variations in the pathogenicity of virus strains.

There is also a difference between the two groups of Coxsackie viruses with respect to growth in tissue culture. Those of group A are, in general, not cultivable in tissue culture, but the A9 type is an exception, and will grow in monkey kidney tissue culture and in some other kinds of tissue culture also, and has frequently been isolated by this method. Some strains of type A9, isolated in tissue culture, have also been found to be nonpathogenic for infant mice, thus resembling the ECHO viruses. Some group B viruses are cultivable in various kinds of tissue culture, but there is some irregularity in the pattern of susceptibility. Monkey kidney tissue culture, prepared in monolayers, appears to be one of the most generally satisfactory for viruses of group B, and cytopathogenic effects are produced.

These viruses are also immunologically heterogeneous and, although there may be some overlapping, there appears to be no common antigenicity to bind these viruses together as a group. They are, rather, immunologically individual.

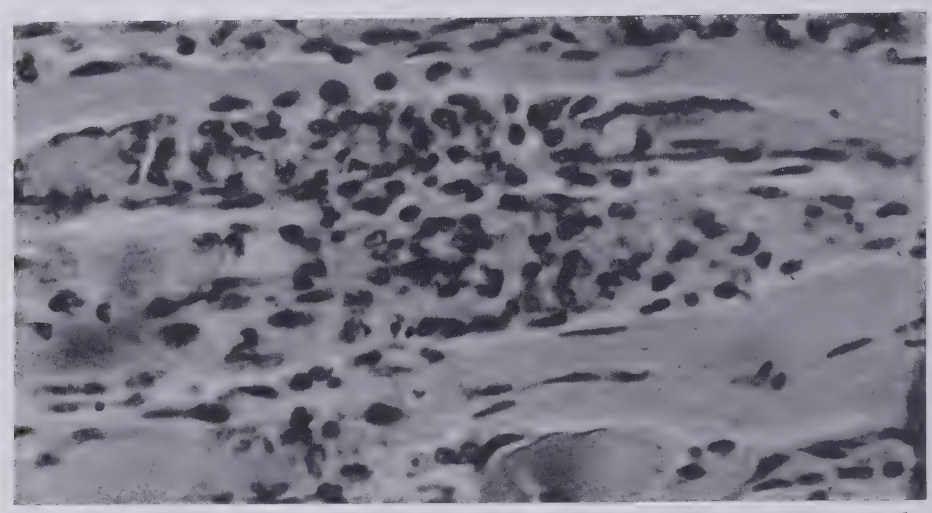


Figure 292. Lesion in striated muscle of a mouse infected with Coxsackie virus. Two sarcolemmic tubes with numerous mononuclear phagocytes and remnants of hyaline material within the sarcolemmic sheaths. Demarcated segmental involvement is apparent. × 500. (Godman, Bunting and Melnick, Amer. J. Pathol.)

A total of nineteen specific serotypes has been defined within group A, and five serotypes within group B, and no doubt additional serotypes will continue to be described.[117] These serotypes are given arbitrarily assigned numbers, to give type designations of A1, A2, etc., and B1, B2, etc.

These types are separable by complement fixation, and by neutralization and protection tests, with substantially the same results. Infected muscle tissue is used for the preparation of immunizing and complement-fixing antigens, and a partial purification of tissue debris may be effected by centrifugation. Protective antibody is demonstrable by the neutralization technique, but infant mice cannot be actively immunized and subsequently challenged because the natural resistance to infection appears very early. An effective passive immunity occurs in infant mice born of and nursed by actively immunized mothers. The protection conferred by active immunity is demonstrable in the chimpanzee as an immunity to challenge inoculation.

Disease in Man. As indicated above, serological evidence indicates a wide prevalence of human infection with Coxsackie viruses, and these viruses have been associated with human illness and found in healthy persons. Their association with poliomyelitis appears to be purely fortuitous, and various attempts to demonstrate a potentiating effect of Coxsackie virus infection on poliomyelitis have given negative results. In fact, an interference between some group B strains and poliovirus occurs in tissue culture, and it has been suggested that Coxsackie virus infection may, on occasion, tend to exclude poliovirus infection.

There are, however, some clinical entities with which Coxsackie viruses have been found to be in close, and possibly etiological, relation. These include febrile pharyngitis and herpangina, epidemic pleurodynia, some portion of cases of aseptic meningitis, and possibly myocarditis in infants. The Coe virus, originally isolated from mild upper respiratory disease, has been found to be Coxsackie A21 virus.[142]

Herpangina. This is a febrile disease, usually seen in children, which occurs in both epidemic and sporadic form in many parts of the world. There is often vomiting and abdominal pain, and the characteristic lesion is a small, herpes-like vesicle which breaks down to leave a shallow ulcer. These lesions occur on the edge of the soft palate, on the tonsils, in the pharynx, and on the anterior pillars of the fauces. Recovery is usually

prompt, within 2 to 3 days, and complete. Coxsackie viruses have been isolated from as many as 84 per cent of cases of herpangina examined, and six serotypes, A2, A4, A5, A6, A8, and A10, have been found for the most part although not exclusively.

There is circumstantial and other evidence, including laboratory infection, which indicates that the group A viruses can produce a febrile pharyngitis, particularly in summer, without the identifying herpangina lesion, giving the disease sometimes known as 3-day fever or summer grippe. When the infection takes this form and occurs during epidemics of poliomyelitis, it may be diagnosed as abortive poliomyelitis. Russian workers have, for example, reported a new type of poliovirus, type 4, which was subsequently shown to be Coxsackie virus type A7.[59, 153] Or, more severe cases of disease have been observed in which the herpangina is associated with parotitis.[61]

Epidemic pleurodynia.[6, 37, 128] This disease was first observed in Bornholm, Denmark, in 1930, and is also known as Bornholm disease, epidemic myalgia, and "devil's grip." The disease tends to occur in epidemic form, most commonly in late summer. The incubation period is 2 to 4 days, the onset sudden, and the disease is characterized by acute pain in the thoracic wall, abdomen, and lower region of the back and is commonly accompanied by headache, but there are no respiratory or constitutional symptoms. The pain is paroxysmal, lasting a day or more, and the muscles are tender to pressure. There is a tendency to relapse at intervals of a few days or longer, and orchitis and meningeal involvement may occur.

Strains of group B Coxsackie virus have been isolated from the throat or feces in a considerable portion of cases in several epidemics studied, increase in antibody titer to the virus isolated is observed, and it seems probable that these viruses may be the etiological agents of this disease.

Aseptic meningitis. Aseptic meningitis is a clinical syndrome rather than an etiological entity and may occur in epidemic form.[14, 127, 150] It is usually mild, with signs and symptoms indicating involvement of the meninges. Fever, headache, nausea, pain in the abdomen, and stiffness of the neck or back usually occur. Pleocystosis may be present, up to 100 cells per cu. mm. being observed in the spinal fluid. This disease may be caused by a variety of nonbacterial agents, including Coxsackie viruses. Viruses of group B, including serotypes B1, B2, B3, and B4, and one of group A, A9 which is set apart from the other group A viruses by its cultivability in monkey kidney tissue as noted above, have been isolated from the feces and from the spinal fluid in cases of this disease. Neutralizing antibody to the isolated virus occurs in the serum, and it is probable that some portion of cases of aseptic meningitis are Coxsackie virus infections.

Myocarditis. In recent years cases of acute myocarditis of infants have been described from various parts of the world. In a few instances isolations of group B Coxsackie viruses have been reported, including recoveries from affected myocardium and from spinal cord. In the latter case, an infant that became ill on the third day after cesarean section and died on the seventh day, serotype B3 virus was found, and the circumstances suggested that intrauterine infection may have occurred.

ENTERIC ORPHAN VIRUSES[25, 65, 101]

With the development of practical and generally applicable methods for the isolation of polioviruses from the intestinal tract in tissue culture, a great many isolations of cytopathogenic agents other than poliovirus have been made. These viruses are previously undescribed entities and, while some have

been found in cases of poliomyelitis, often diagnosed as the nonparalytic disease, in many instances there has been no specific association with illness, and they have been called orphan viruses. Those isolated from man have been named the ECHO, or enteric cytopathogenic human orphan, viruses, those from monkeys the ECMO viruses, those from cattle the ECBO viruses, and those from swine the ECSO viruses. Similar viruses have been isolated from dogs which may make up another group of ECDO viruses. Of these, the ECHO viruses have been of the greatest interest, and, with their characterization, some at least have been found to occur in association with, and possibly etiological relation to, human disease.

ECHO VIRUSES

These viruses are similar to polioviruses and Coxsackie viruses in size and have been found to be about 30 mμ in diameter with the exception of one, ECHO type 10, which has been reported to be 60 to 90 mμ in diameter. They are differentiated from one another by their host range and pathogenicity as expressed in tissue culture, including plaque morphology in monolayer culture, and by separation into antigenic types. Some, though not all, form hemagglutinin and are related to one another in this respect, but apparently not to the influenza and other myxoviruses.

Tissue Culture. In general, the ECHO viruses grow more readily on primary isolation in monkey kidney tissue culture than on HeLa cell culture. This is in contrast to the adenoviruses which are usually more readily isolated in HeLa cell culture. Many strains of ECHO viruses have been grown in HeLa cell culture, with the appearance of cytopathogenic effects and complement-fixing antigen in the culture fluid, but the virus titer reached is not as high as that in monkey kidney tissue culture.[5, 103]

Plaque morphology. Growth in kidney tissue culture from a variety of species of monkeys has been studied in detail[63, 64, 66] as has the comparative susceptibility of the cells to these, and to poliomyelitis and Coxsackie viruses, assayed by the rate of development and morphology of plaques produced in monolayer cultures. It will be recalled that host cell susceptibility and plaque morphology have been established as valid differential characteristics, inheritable and genetically determined, in the case of bacterial viruses (Chaps. 3 and 7), and may be regarded as analogous to the morphology of pocks produced on the chorioallantoic membrane by poxviruses and to bacterial colonial morphology on differential mediums. Of the various species of monkeys investigated, the kidney tissue of two, the rhesus and patas (*Erythrocebus patus*, the African red grass monkey), have been of considerable differential value.

On monolayers of rhesus cells, the polioviruses produce circular plaques

Differentiation of Enteric Viruses in Tissue Culture

VIRUS		SEROTYPE	PLAQUE MORPHOLOGY	PLAQUE FORMATION	
				RHESUS CELLS	PATAS CELLS
poliomyelitis		1, 2, 3	large, circular, sharp boundaries	+	+
Coxsackie	B	1, 2, 3, 4, 5	large, circular,		
	A	9	diffuse boundaries, slow development	+	–
ECHO	A	1, 3, 4, 6′, 9, 11, 13, 14	small or medium size, irregular, slow development	+	–
	B	7, 8, 12	large, circular	+	+

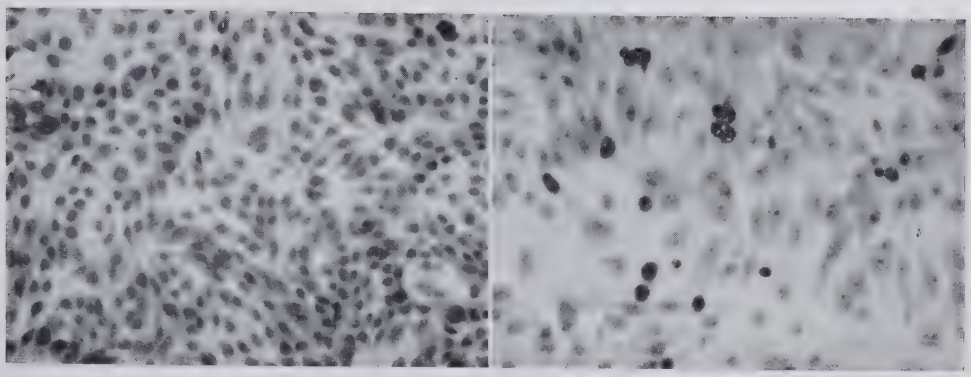


Figure 293. The cytopathogenic effect of ECHO virus type 1 in culture of monkey kidney epithelial cells. *Left*, uninoculated 6-day culture of cells; *right*, 24 hours after the inoculation of virus, showing a few rounded cells scattered throughout the field. Carnoy fixation, hematoxylin and eosin. (Melnick, Adv. Virol.)

with clear centers and sharp boundaries as illustrated in the accompanying figure. The plaques formed by the Coxsackie viruses which are cultivable in tissue culture, group B and A9, develop more slowly, and the boundaries are not sharp owing to the persistence of living cells at the periphery. The plaque-producing ECHO viruses are separable into two groups. One, designated group A and containing serotypes 1, 3, 4, 6′, 9, 11, 13, and 14, produces slowly developing, small to medium size, irregular plaques, and group B, consisting of serotypes 7, 8, and 12, produces large circular plaques.

The susceptibility of the two kinds of monolayer kidney cell cultures is also differential. Poliovirus and ECHO group B form plaques on both kinds of cells, but the Coxsackie viruses and ECHO group A form plaques on rhesus cell cultures but not on patas cell cultures. There is further differential susceptibility within the ECHO viruses in that serotypes 2, 3, 5, and 6 do not produce plaques on either kind of cells, and they are cytopathogenic for rhesus cells but not for patas cells in tube cultures with fluid overlay.

Antigenic Types. The ECHO viruses are separable into serotypes by cross-neutralization tests in tissue culture and standard antiserums are used for identification.[74] The test is made sharply specific by titration of the 50 per cent tissue culture dose, the TCD_{50}, and the prototype strains have been numbered on an arbitrary basis. New serotypes continue to be found; for example, as noted elsewhere (Chap. 38), the JH and 2060 respiratory viruses are now identified as ECHO 28, and the recently described Frater virus, recovered from aseptic meningitis, appears to be a new ECHO type.[35, 36] Strains within a serotype are not necessarily antigenically identical, and those with broader antigenicity than the prototype strain have been called prime strains. Strains within type 6 have been studied in this connection, and the strains designated 6′ and 6″ have been found to have a broader antigenic specificity than that of prototype strain 6. There are cross-reactions among types 1, 8 and 12, and ECHO 4 antiserum neutralizes adenovirus 8, and Coxsackie B4 is neutralized by antiserums to several ECHO types.

Reoviruses.[132] As noted above ECHO 10 is set apart from the other viruses of this group in that it is considerably larger. It forms hemagglutinin for human O cells but not for chicken, guinea pig, sheep or bovine erythrocytes; the red cell receptors are not affected by RDE but are destroyed by treatment with periodate. There is a distinctive cytopathic effect in monkey kidney cell culture, with granular degeneration of the cells leaving intact nuclei, and intracytoplasmic inclusion bodies are present. These and other distinctive characteristics[62] have led to the separation of

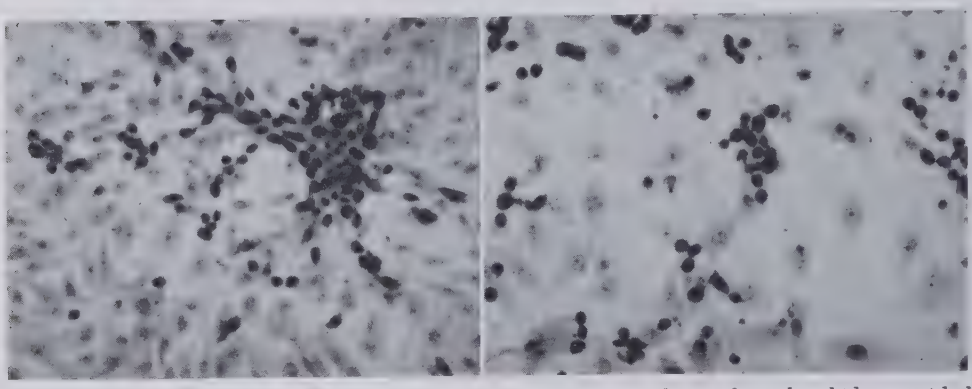


Figure 294. The cytopathogenic effect of ECHO virus type 1 in culture of monkey kidney epithelial cells. *Left,* 66 hours after the inoculation of virus; *right,* 90 hours after the inoculation of virus. At 66 hours there are foci of rounded, densely stained cells and infected cells scattered over the cell sheet. At 90 hours many infected cells have become detached from the glass, most of the remaining cells are rounded because of viral infection, but some normal-appearing cells are still present. Carnoy fixation, hematoxylin and eosin. (Melnick, Adv. Virol.)

ECHO 10 strains as a separate group designated reoviruses, the term referring to both respiratory and enteric origin.

Strains of this group contain a common, or group-specific, complement-fixing antigen, and are separable into serotypes by neutralization and hemagglutination-inhibition tests.[124] Three serotypes are distinguished. Type 1 consists of the Lang strain, found in healthy children, as the prototype and includes the SV-12 simian virus, and other strains isolated from children with upper respiratory disease. Type 2 includes the D5 strain, found in a child with diarrhea, as the prototype, together with simian strains and others of human origin. Two strains have been suggested as the prototype of type 3, one the Dearing strain from a child with diarrhea and the other the Abney strain recovered from a child with febrile upper respiratory disease[125] and subsequently from nursery children. Type 3 strains have also been recovered from cattle.[126] Types 2 and 3 have been found in wild and laboratory mice respectively.[53] Suckling mice may be infected experimentally, some strains producing clinical disease.

Pathogenicity.[137] In a general way, the ECHO viruses are set apart from the Coxsackie viruses in their lack of pathogenicity for the infant mouse although, as noted above, occasional Coxsackie strains are not pathogenic for the infant mouse on primary isolation. While originally found in the absence, or apparent absence, of specific disease, certain of the ECHO viruses have been found in association with human disease, especially aseptic meningitis.

Types 4, 5, 6, 9, and 16 have been found repeatedly in cases of aseptic meningitis in the absence of other causative agents, and types 5, 6, and 9 have been isolated from the spinal fluid.[30, 91, 115] Epidemic aseptic meningitis occurred in Europe in 1955 and 1956, and spread to this country in the following year, in which type 9 appeared to be the etiological agent. The virus strains found were apparently antigenic variants of the prototype strain. Infections associated with type 9, and also with types 4 and 16, have often been characterized also by the presence of a rash. In view of its association with aseptic meningitis, the status of ECHO type 9 is of interest; certain of the apparently more virulent strains, *i.e.*, isolated from spinal fluid, have been found to become pathogenic for infant mice after tissue culture passage, producing myositis and paralysis, and might be considered to be a type B Coxsackie virus. In general, the evidence is sufficiently scanty that the pathogenicity of the ECHO viruses for man remains somewhat uncertain.

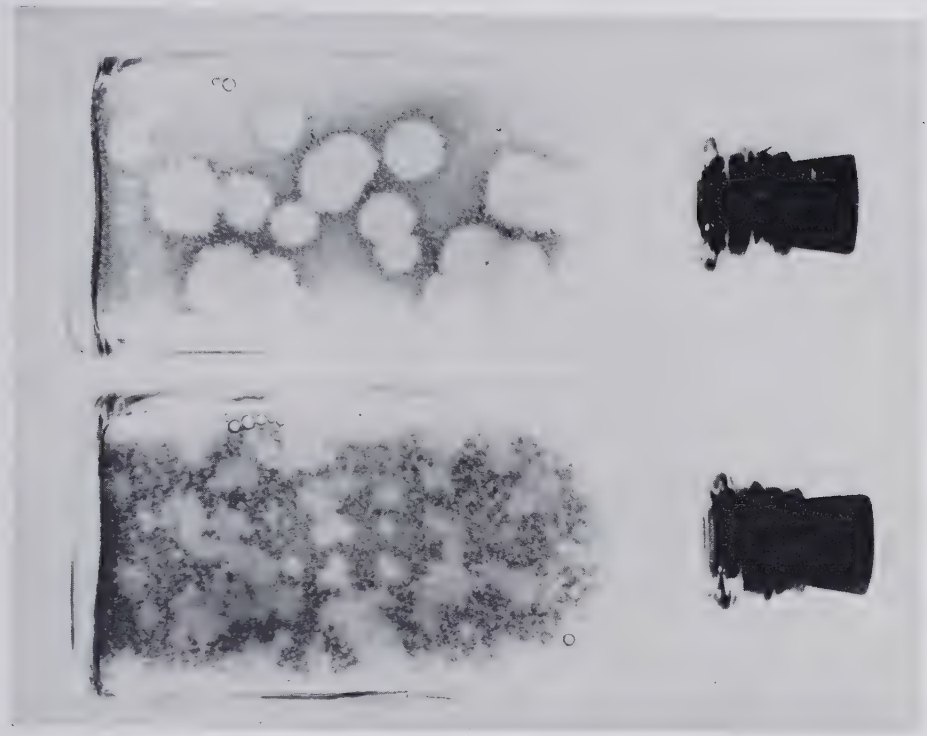


Figure 295. The types of plaques produced by poliovirus type 3 (*above*) and ECHO virus type 6′ (*below*) on monolayer cultures of monkey kidney tissue. (Hsuing and Melnick, Virology.)

ENTERIC ORPHAN VIRUSES OF LOWER ANIMALS[73]

It was noted above that viruses analogous to the ECHO viruses have been found in lower animals, including monkeys, cattle, and swine. The simian viruses have been of most interest since they are found both in the bowel[23] and in apparently healthy monkey kidney tissue[68, 69] used for culture of the poliomyelitis and other viruses. A considerable number of simian strains have been examined for plaque morphology on rhesus and patas monkey kidney tissue culture and for antigenic specificity.[65] On the first basis they are separable into three groups: group A characterized by the delayed development of small plaques on both rhesus and patas cultures; group B made up of strains forming large circular plaques on both rhesus and patas cultures in which islets of unaffected cells are found; and group C strains which produce plaques on rhesus cultures similar to those of group B but do not produce plaques on patas cultures. Differentiation on the basis of plaque morphology is not necessarily consistent with antigenic differences, and cross-neutralization is observed between plaque types. So far as is known, these viruses, the ECMO group, are not pathogenic for monkeys.

VIRAL DIARRHEAS

Diarrheal disease of viral etiology occurs in both man and lower animals. ECHO virus type 18 has been found to be associated with summer diarrhea of infants.[38] Other epidemic diarrhea of the newborn may also be of viral etiology, and there is some evidence suggesting that the disease may be transmitted to

calves, but the causative agent is poorly known.

Viral diarrhea, or epidemic nonbacterial gastroenteritis, occurs in adults also and has been studied at length by Gordon and his colleagues.[49] It has been found that at least two kinds of disease occur, a febrile and an afebrile type. The afebrile type has an incubation period of 2 to 3 days and is characterized by nausea, vomiting, abdominal spasms, and watery stools, and an attack results in an immunity which persists for a year or more. It is associated with a filterable agent, Marcy, which has been transferred through eight serial passages in human volunteers, but disease has not been produced in experimental animals. The agent associated with the febrile disease, which has a shorter incubation period and a less protracted course, has been designated FS, and there is evidence that this and the Marcy agent are immunologically distinct. The Marcy agent appears to be closely related to, if not identical with, the agent responsible for nonbacterial infectious diarrhea in Japan.[43]

Diarrheal disease of viral etiology is not uncommon in lower animals. One of the most familiar of these to the laboratory worker is the epidemic diarrhea of suckling mice which occurs in explosive form, more commonly in the winter months. Intranuclear inclusion bodies are found in the epithelial cells of the villi, but the nature of the etiological agent is not well established. Calf pneumonia enteritis is of established viral etiology, the virus has been isolated, and the disease transmitted to susceptible newborn animals.[13] The psittacosis virus may cause diarrheal disease in older animals, and three other immunologically distinct agents, presumably viral in nature, may also cause the disease. Transmissible gastroenteritis of swine is also of viral etiology, and the virus has been isolated and characterized immunologically.[51]

VIRAL HEPATITIS[39, 46, 55]

Liver damage with icterus occurs in infectious diseases such as leptospirosis and yellow fever, but there is, in addition, hepatitis of specific viral etiology. There are two kinds of this disease, the one infectious hepatitis and the other serum hepatitis, which are caused by different viruses and differ in epidemiological character.

INFECTIOUS HEPATITIS

Infectious hepatitis (infective hepatitis, epidemic jaundice) is a subacute disease of world-wide distribution which tends to occur in children and young adults. It may appear in epidemic form within households and institutions and in military personnel, as in the two World Wars and the war in Korea. The prevalence of the infection is not precisely known, and it is probable that many cases are subclinical.

The incubation period varies from 1 to 6 weeks, with an average of perhaps 3 to 4 weeks. The disease is one of diffuse involvement of the liver and is separable into two stages, the pre-icteric and the icteric. The onset may be abrupt or insidious, and the symptoms include fever, gastrointestinal distress, headache, anorexia, and lassitude; the postcervical lymph nodes are often involved, and there is leucopenia with relative lymphocytosis. This pre-icteric phase may persist for as long as 3 weeks but usually lasts for only a few days. Symptoms tend to subside, but there is exacerbation of some, especially those of abdominal discomfort, with the appearance of jaundice, and the liver and spleen are palpable and tender. Examination of biopsy

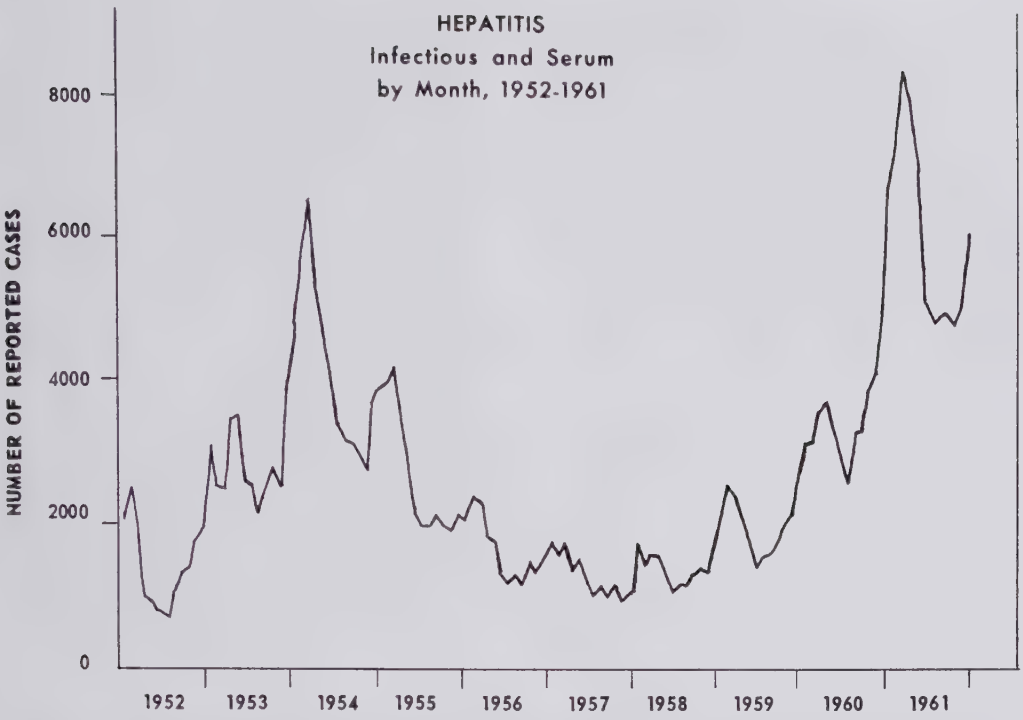


Figure 296. Reported cases of hepatitis, both infectious and serum, in the United States, 1952–1961. Reproduced from the Morbidity and Mortality Weekly Report Annual Supplement, Vol. 10, No. 53. Communicable Disease Center, Public Health Service, U. S. Department of Health, Education and Welfare.

specimens indicates progressive liver damage during the course of the disease, with relatively extensive parenchymal destruction by the time jaundice is apparent, but subsequently regeneration occurs which is complete in most cases by 3 months. Consistent with this, there is early evidence of hepatic dysfunction, and bilirubinuria occurs toward the end of the pre-icteric phase of the disease.

The icteric phase may persist for some weeks and is usually followed by uneventful convalescence. The case fatality rate is lower in children than in adults and has been reported as less than 2 per 1000 in military personnel. Relapse occurs with variable frequency, however, ranging from less than 1 per cent to as much as 18 per cent of cases. In many instances relapse is evident only as some degree of hepatic dysfunction, but in others the icteric phase of the disease recurs, although often in less severe form. In some few cases impairment of hepatic function may persist over protracted periods, and in some small portion of these, symptoms may persist also. Rare complications of the disease include pneumonia, meningitis, and myelitis.

Hepatitis Virus A. It is customary to designate the virus of infectious hepatitis as hepatitis virus A to distinguish it from the virus of serum hepatitis. It has not been possible as yet to infect experimental animals although there is evidence suggesting that chimpanzees may have been a source of human infection.[56, 120] There are a good many reports of isolation of a viral agent in embryonated eggs or one or another kind of tissue cell culture,[31, 57, 102, 113, 121] none of which has as yet been accepted as an etiological agent of the disease. Information is limited to that obtained from the inoculation of human volunteers.

The virus passes bacteria-proof filters such as the Seitz EK and is relatively resistant, for it is not inactivated at 56° C. for 30 minutes or by concentrations as high as 1 ppm of residual chlorine in the

same time. It is present in the blood and feces in the pre-icteric or early icteric stages of the disease and has been found in the feces during the incubation period, as much as 2 to 3 weeks before the onset of jaundice,[81, 154] but attempts to demonstrate the virus in urine and in nasopharyngeal secretions have given equivocal or negative results. Man may be infected by either parenteral inoculation or ingestion of the virus, and it is probable that the latter route is involved in most naturally-occurring infections.[99, 112, 116] Water-borne, milk-borne, and food-borne epidemics have been described, and other more direct forms of contact are indicated by epidemiological evidence. The occurrence of the disease in the winter months suggests respiratory spread of the infection, but this is not consistent with transmission of the disease by biting insects even though the blood is highly infectious.

The relatively greater prevalence and severity of the disease in the lower age groups, including young adults, suggests that the infection may be relatively prevalent and produces an effective immunity. Pooled γ-globulin has a definite protective effect when given during the incubation period. Effective immunity has also been demonstrated experimentally with human volunteers, but there is some reason to believe that all strains of the virus are not immunologically identical although cross-protection occurs between strains. Hepatitis virus A is antigenically distinct from the virus of serum hepatitis. Second attacks of hepatitis occur, but the lack of a specific diagnostic test makes it difficult to assess their significance.

SERUM HEPATITIS

The disease serum hepatitis has probably been prevalent for many years, and possibly epidemics of jaundice associated with vaccination and the chemotherapy of syphilis were of this nature. It came into prominence in the early 1940's in connection with the immunization of military personnel to yellow fever with an immunizing agent which included human serum. Jaundice appeared to be associated with some, but not all, lots of serum, and with the elimination of serum from the vaccine, cases of so-called postvaccinal jaundice ceased to occur. Subsequently, there have been many instances of icteric disease following the injection of human serum or plasma, or blood transfusions, all indicating the presence of an infectious agent in human blood.

The incubation period of serum hepatitis is relatively long, 8 to 22 weeks, and the disease is indistinguishable clinically from infectious hepatitis after the onset of symptoms. It has not been possible to transmit this disease to experimental animals, and information about the virus has been derived from experimental inoculation of human volunteers.

Hepatitis Virus B. The causative virus is named hepatitis virus B to distinguish it from hepatitis virus A of infectious hepatitis. The two viruses have similar properties in that both show substantially the same resistance to heating, and activity persists in blood, serum, and plasma over extended periods. They are, however, immunologically distinct, there appears to be no cross-immunity between them, and they may be found together in both place and time. Hepatitis virus B is present in the blood, not only during the disease, but also in the incubation period, and a chronic carrier state has been observed in which the blood may be infective for many months. It is not found in nasopharyngeal secretions, and it differs from hepatitis virus A in that it is not present in the feces, and man is not infected experimentally by the gastrointestinal route.

It is quite uncertain how the infection persists under natural conditions, but it is readily transmitted to man by inadequately sterilized syringes and needles used for purposes such as the administration of chemotherapeutic drugs, convalescent serum, or insulin or withdrawing blood, by procedures such as tattooing, and by the administration

of infectious blood, plasma, or certain blood products such as fibrin foam and thrombin. Hepatitis virus A may, of course, also be transmitted in this way.

As in infectious hepatitis, antibody concentrates such as γ-globulin are prophylactic, and an attack of the disease appears to confer an appreciable degree of effective immunity. The virus is rarely found in γ-globulin; such preparations are possibly contaminated, but the virus may be neutralized by the relatively high concentration of antibody.

The persistence of the virus in blood, plasma, and serum poses a considerable practical problem in view of the lack of any method, other than human inoculation, of demonstrating its presence. It is destroyed by ultraviolet irradiation under experimental conditions, but this is not a practical method for the treatment of blood or plasma, and chemical sterilization, as by nitrogen mustards, has not yet been successful. Allen and his co-workers[3, 4] have found that the incidence of hepatitis in recipients of plasma is markedly reduced when the fluid plasma is allowed to stand at room temperature for 3 to 6 months, suggesting an appreciable degree of inactivation of the virus.

HEPATITIS OF LOWER ANIMALS

Hepatitis of viral etiology occurs in lower animals, including the dog and mouse, and a similar disease has been described in guinea pigs and ducks.[72] Murine hepatitis has been of interest as a disease possibly analogous to viral hepatitis of man.

Canine Hepatitis. An infectious hepatitis of dogs was described in 1947 by Rubarth in Sweden as caused by a virus related to the fox encephalitis virus; it is sometimes known as Rubarth's disease. The disease ranges in severity from symptomless infections to an acute fulminating fatal disease, with death occurring within 24 hours. The disease may be reproduced in susceptible puppies, but not in the usual experimental animals. Intranuclear inclusion bodies are found in the liver cells and vascular endothelium, and the virus passes bacteria-proof filters. The virus has been cultivated in the yolk sac of embryonated eggs and in raccoon kidney tissue culture,[98] reproducing the disease in susceptible puppies after twelve passages in the former and after thirty-nine passages in the latter. A lyophilized antigen prepared from infectious liver tissue fixes complement in the presence of serum from convalescent dogs,[84, 89] and Rubarth found that the majority of adult dog serums contain complement-fixing antibody, suggesting that the infection is widespread and usually symptomless. This inference is substantiated by the isolation of the virus from dog kidney tissue cultures derived from two apparently healthy dogs.[12]

Human infection with the canine virus has been reported,[141] but there is apparently no relation between the human and canine viruses.[7] There is a one-sided antigenic relationship between certain adenoviruses and the canine hepatitis virus in that the latter will fix complement in the presence of antibody to the former, a relationship substantiated by the passive hemagglutination and gel-diffusion techniques.[17, 75] The canine virus resembles the adenoviruses in being ether-resistant, producing similar cytopathic effects, and is of the same order of size.

Murine Hepatitis.[111] Naturally occurring murine hepatitis was described in 1951 in England by Gledhill and Andrewes. The original disease was complicated by the presence of *Eperythrozoon coccoides,* but a fulminating infection, lethal in about 1 week, can be produced in the newborn mouse which is characterized by extensive degenerative lesions in the liver. The adult mouse is not susceptible to the disease.

The virus is present in the blood, urine, and feces and is infectious by the gastrointestinal as well as parenteral routes of inoculation. It has not been possible to obtain clear-cut evidence of growth of the virus in the embryonated

egg. Inoculation with relatively large doses of virus results in the appearance of an increased number of monocytes in the chorioallantoic fluid, an effect similar to that observed with human hepatitis virus, but the infection could not be passed in eggs. The virus multiplies in mouse macrophage cultures, but no cytopathic effect occurs and the presence of the virus is indicated only by comparative cell counts. More recently the MHV3 strain of the virus has been propagated serially in fetal mouse liver explants on rat-tail reconstituted collagen to produce cytopathic changes, largely in the epithelial outgrowth.[151] It has been found too that the MHV-(Babl D) strain multiplies in mouse embryo cell cultures and, after passage, in mouse kidney epithelium and L cell culture, giving a cytopathic effect in all three systems.[100]

REFERENCES

1. Ackermann, W. W., Payne, F. E., and Kurtz, H.: Concerning the cytopathogenic effect of poliovirus; evidence for an extraviral toxin. J. Immunol., 1958, **81:**1–6.
2. Akers, T. G., Hashem, N., and Mahdy, M. S.: Studies on paralytic poliomyelitis in Cairo, U. A. R., 1957–1958. Amer. J. Trop. Med. Hyg., 1960, **9:**343–349.
3. Allen, J. G., et al.: Homologous serum jaundice and its relation to methods of plasma storage. J. Amer. Med. Assn., 1950, **144:** 1069–1074.
4. Allen, J. G., Enerson, D. M., Barron, E. S. G., and Sykes, C. J.: Pooled plasma with little or no risk of homologous serum jaundice. J. Amer. Med. Assn., 1954, **154:**103–107.
5. Archetti, I., Weston, J., and Wenner, H. A.: Adaptation of ECHO viruses in HeLa cells, their use in complement fixation. Proc. Soc. Exptl. Biol. Med., 1957, **95:**265–270.
6. Bain, H. W., McLean, D. M., and Walker, S. J.: Epidemic pleurodynia (Bornholm disease) due to Coxsackie B-5 virus. The interrelationship of pleurodynia, benign pericarditis and aseptic meningitis. Pediatrics, 1961, **27:**889–903.
7. Bech, V.: Canine hepatitis virus: Attempts to find relationship with human hepatitis. Proc. Soc. Exptl. Biol. Med., 1959, **100:**135–137.
8. Berkovich, S., Pickering, J. E., and Kibrick, S.: Paralytic poliomyelitis in Massachusetts, 1959. A study of the disease in a well vaccinated population. New England J. Med., 1961, **264:**1323–1329.
9. Bodian, D.: Viremia, invasiveness, and influence of injections. Ann. N. Y. Acad. Sci., 1955, **61:**877–882.
10. Bodian, D.: Emerging concept of poliomyelitis infection. Science, 1955, **122:**105–108.
11. Bodian, D.: Poliovirus in chimpanzee tissues after virus feeding. Amer. J. Hyg., 1956, **64:** 181–197.
12. Bolin, V. S., Jarnevic, N., and Austin, J. A.: Infectious canine hepatitis virus studies with special reference to passage in raccoon tissue cultures. Proc. Soc. Eptl. Biol. Med., 1958, **98:**414–418.
13. Brandly, C. A., and McClurkin, A. W.: Epidemic diarrheal disease of viral origin of newborn calves. Ann. N. Y. Acad. Sci., 1956, **66:**181–185.
14. Brohl, I., Helmstaedt, K., Lennartz, H., and Maass, G.: Über eine Epidemie von Coxsackie-Meningitis. Ztschr. f. ärztl. Fortbildung, 1957, **51:**499–505.
15. Cabasso, V. J., et al.: Cumulative testing experience with consecutive lots of oral poliomyelitis vaccine. Brit. Med. J., 1960, **i:**373–387.
16. Cabasso, V. J., et al.: Oral poliomyelitis vaccine, Lederle—Thirteen years of laboratory and field investigation. An interim review. New England J. Med., 1960, **263:**1321–1330.
17. Carmichael, L. E., and Barnes, F. D.: Serological comparisons between infectious canine hepatitis virus and human adenovirus types. Proc. Soc. Exptl. Biol. Med., 1961, **107:**214–218.
18. Casey, A. E., Fishbein, W. I., Schabel, F. M., and Smith, H. T.: Incidence of subclinical poliomyelitis in an urban area according to age groups. Amer. J. Pub. Health, 1950, **40:** 1241–1250.
19. Chadwick, D. L., Welsh, H. H., and Lennette, E. H.: Comparison of the sensitivity of several mammalian cell types for the isolation of poliomyelitis viruses from man. J. Lab. Clin. Med., 1959, **54:**445–450.
20. Chaproniere, D. M., Done, J. T., and Andrewes, C. H.: Comparative serological studies on talfan and teschen diseases and similar conditions. Brit. J. Exptl. Path., 1958, **39:**74–77.
21. Charney, J., Fischer, W. P. M., Sagin, J. F., and Tytell, A. A.: Inactivation of concentrated purified poliovirus suspensions. Ann. N. Y. Acad. Sci., 1960, **83:**649–653.
22. Charney, J., Tytell, A. A., Machlowitz, R. A., and Hilleman, M. R.: Development of a purified poliomyelitis vaccine. J. Amer. Med. Assn., 1961, **177:**591–595.
23. Cheever, F. S.: Discussion of viral agents of the gastrointestinal tracts of monkeys. Ann. N. Y. Acad. Sci., 1957, **67:**427–429.
24. Chumakov, M. P., et al.: Some results of the work on mass immunization in the Soviet Union with live poliovirus vaccine prepared from Sabin strains. Bull. W. H. O., 1961, **25:**79–91.
25. Committee: Enteric cytopathogenic human orphan (ECHO) viruses. Science, 1955, **122:** 1187–1188.
26. Cooper, P. D.: An improved agar cell-suspension plaque assay for poliovirus: Some factors affecting efficiency of plating. Virology, 1961, **13:**153–157.
27. Cox, H. R.: Oral poliomyelitis vaccine. Bact. Rev., 1961, **25:**383–388.
28. Cox, H. R., et al.: Immunization against poliomyelitis with a trivalent oral vaccine. Patho-

logia Microbiol., 1961, **24:** Suppl., pp. 61–72.
29. Dauer, C. C.: The changing age distribution of paralytic poliomyelitis. Ann. N. Y. Acad. Sci., 1955, **61:**943–955.
30. Davis, D. C., and Melnick, J. L.: Association of ECHO virus type 6 with aseptic meningitis. Proc. Soc. Exptl. Biol. Med., 1956, **92:**839–843.
31. Davis, E. V.: Isolation of viruses from children with infectious hepatitis. Science, 1961, **133:** 2059–2061.
32. Debre, R., et al.: Poliomyelitis. W. H. O. Monograph Series No. 26. World Health Organization, Palais des Nations, Geneva. 1955.
33. Dubes, G. R., Archetti, I., and Wenner, H. A.: Antigenic variants among type 3 polioviruses. Amer. J. Hyg., 1959, **70:**91–105.
34. Dulbecco, R., and Vogt, M.: Biological properties of poliomyelitis viruses as studied by the plaque method. Ann. N. Y. Acad. Sci., 1955, **61:**790–800.
35. Duncan, I. B. R.: Aseptic meningitis associated with a previously unrecognized virus. Lancet, 1960, **ii:**470–471.
36. Duncan, I. B. R.: Biological and serological properties of Frater virus—a cytopathogenic agent associated with aseptic meningitis. Arch. f. d. gesamte Virusf., 1961, **11:**248–257.
37. Duxbury, A. E., and Warner, P.: Epidemiological and laboratory investigations on Bornholm disease in Adelaide, 1957. Med. J. Australia, 1958, **19:**518–523.
38. Eichenwald, H. F., Ababio, A., Arkey, A. M., and Hartman, A. P.: Epidemic diarrhea in premature and older infants caused by ECHO virus type 18. J. Amer. Med. Assn., 1958, **166:**1563–1566.
39. Eichenwald, H. F., and Mosley, J. W.: Viral hepatitis. Clinical and public health aspects. Public Health Service Publication No. 435 (revised). U. S. Department of Health, Education and Welfare. 1959.
40. Enders, J. F., Weller, T. H., and Robbins, F. C.: Alteration in pathogenicity for monkeys of Brunhilde strain of poliomyelitis virus following cultivation in human tissues. Fed. Proc., 1952, **11:**467.
41. Fox, J. P., Gelfand, H. M., LeBlanc, D. R., and Rowan, D. F.: The influence of natural and artificially induced immunity on alimentary infections with polioviruses. Amer. J. Pub. Health, 1958, **48:**1181–1192.
42. Francis, T., Jr., et al.: An evaluation of the 1954 poliomyelitis vaccine trials. Amer. J. Pub. Health, 1955, **45:**No. 5, Pt. 2, pp. 1–63.
43. Fukumi, H., et al.: An indication as to the identity between the infectious diarrhea in Japan and the afebrile infectious nonbacterial gastroenteritis by human volunteer experiments. Japanese J. Med. Sci. Biol., 1957, **10:**1–17.
44. Gard, S.: Inactivation of poliovirus by formaldehyde. Theoretical and practical aspects. Bull. W. H. O., 1957, **17:**979–989.
45. Gelfand, H. M.: The occurrence in nature of the Coxsackie and ECHO viruses. Progress Med. Virol., 1961, **3:**193–244.
46. Gellis, S. S., and Hsia, D. Y.: Viral hepatitis (a review). New England J. Med., 1953, **249:**400–409.
47. Gerfeldt, E.: Von Erscheinungswandel der Poliomyelitis. Zur Epidemiologie und Sozialpathologie der Poliomyelitis in Deutschland. Arch. f. Hyg. u. Bakt., 1960, **144:**442–451.
48. Gildemeister, H., et al.: Praktikum der Virusdiagnostik. Gustav Fischer Verlag, Stuttgart. 1961.
49. Gordon, I., and Whitney, E.: Virus diarrheas of adults and their possible relationships to infantile diarrhea. Ann. N. Y. Acad. Sci., 1956, **66:**220–225.
50. Greenberg, M., Abramson, H., Cooper, H. M., and Solomon, H. E.: The relation between recent injections and paralytic poliomyelitis in children. Amer. J. Pub. Health, 1952, **42:** 142–152.
51. Haeltermann, E. O., and Hutchings, L. M.: Epidemic diarrheal disease of viral origin in newborn swine. Ann. N. Y. Acad. Sci., 1956, **66:**186–190.
52. Hammon, W. McD., Coriell, L. L., and Stokes, J., Jr.: Evaluation of Red Cross gamma globulin as a prophylactic agent for poliomyelitis. I. Plan of controlled field tests and results of 1951 pilot study in Utah. J. Amer. Med. Assn., 1952, **150:**739–749.
53. Hartley, J. W., Rowe, W. P., and Huebner, R. J.: Recovery of reoviruses from wild and laboratory mice. Proc. Soc. Exptl. Biol. Med., 1961, **108:**390–395.
54. Hatch, M. H., Kalter, S. S., and Ajello, G. W.: Identification of poliovirus isolates with fluorescent antibody. Proc. Soc. Exptl. Biol. Med., 1961, **107:**1–4.
55. Havens, W. P., Jr.: Hepatitis, yellow fever, and dengue. Ann. Rev. Microbiol., 1954, **8:**289–310.
56. Hillis, W. D.: An outbreak of infectious hepatitis among chimpanzee handlers at a United States Air Force base. Amer. J. Hyg., 1961, 73:316–328.
57. Hillis, W. D.: Destruction of chimpanzee kidney cells by sera from patients with acute infectious hepatitis. Proc. Soc. Exptl. Biol. Med., 1961, **108:**813–816.
58. Holguin, A. H., and Reeves, J. S.: Immunization of infants with the Sabin oral poliovirus vaccine. Amer. J. Pub. Health, 1962, **52:** 600–610.
59. Horstmann, D. M., and Manuelidis, E. E.: Russian Coxsackie A-7 virus ("AB IV" strain)—neuropathogenicity and comparison with poliovirus. J. Immunol., 1958, **81:** 32–42.
60. Horstmann, D. M., Niederman, J. C., and Paul, J. R.: Attenuated type 1 poliovirus vaccine. Its capacity to infect and to spread from "vaccinees" within an institutional population. J. Amer. Med. Assn., 1959, **170:** 1–8.
61. Howlett, J. G., Somlo, F., and Kale, F.: A new syndrome of parotitis with herpangina caused by the Coxsackie virus. Canadian Med. Assn. J., 1957, **77:**5–7.
62. Hsuing, G. D.: Some distinctive biological characteristics of ECHO-10 virus. Proc. Soc. Exptl. Biol. Med., 1958, **99:**387–390.
63. Hsuing, G. D., and Melnick, J. L.: Morphologic characteristics of plaques produced on monkey kidney monolayer cultures by enteric viruses (poliomyelitis, Coxsackie and ECHO groups). J. Immunol., 1957, **78:** 128–136.
64. Hsuing, G. D., and Melnick, J. L.: Comparative susceptibility of kidney cells from dif-

ferent monkey species to enteric viruses (poliomyelitis, Coxsackie and ECHO groups). J. Immunol., 1957, **78:**137–146.
65. Hsuing, G. D., and Melnick, J. L.: Orphan viruses of man and animals. Ann. N. Y. Acad. Sci., 1958, **70:**342–361.
66. Hsuing, G. D., and Melnick, J. L.: Adsorption, multiplication and cytopathogenicity of enteroviruses (poliomyelitis, Coxsackie and ECHO groups) in susceptible and resistant monkey kidney cells. J. Immunol., 1958, **80:**45–52.
67. Huebner, R. J., et al.: The importance of Coxsackie viruses in human disease particularly herpangina and epidemic pleurodynia. New England J. Med., 1952, **247:**249–256; 285–289.
68. Hull, R. N., and Minner, J. R.: New viral agents recovered from tissue cultures of monkey kidney cells. II. Problems of isolation and identification. Ann. N. Y. Acad. Sci., 1957, **67:**413–423.
69. Hull, R. N., Minner, J. R., and Smith, J. W.: New viral agents recovered from tissue cultures of monkey kidney cells. I. Origin and properties of cytopathogenic agents SV_1, SV_2, SV_4, SV_5, SV_6, SV_{11}, SV_{12}, and and SVL_5. Amer. J. Hyg., 1956, **63:**204–215.
70. Hummeler, K., and Hamparian, V. V.: Studies on the complement fixing antigens of poliomyelitis. I. Demonstration of type and group specific antigens in native and heated viral preparations. J. Immunol., 1958, **81:**499–505.
71. Hummeler, K., and Tumilowicz, J. J.: Studies on the complement fixing antigens of poliomyelitis. II. Preparation of type-specific anti-N and anti-H indicator sera. J. Immunol., 1960, **84:**630–634.
72. Kaeberle, M. L., Drake, J. W., and Hanson, L. E.: Cultivation of duck hepatitis virus in tissue culture. Proc. Soc. Exptl. Biol. Med., 1961, **106:**755–757.
73. Kalter, S. S.: Animal "orphan" enteroviruses. Bull. W. H. O., 1960, **22:**319–337.
74. Kamitsuka, P. S., Soergel, M. E., and Wenner, H. A.: Production and standardization of ECHO reference antisera. I. For 25 prototypic ECHO viruses. Amer. J. Hyg., 1961, **74:**7–25.
75. Kapsenberg, J. G.: Relationship of infectious canine hepatitis virus to human adenovirus. Proc. Soc. Exptl. Biol. Med., 1959, **101:**611–614.
76. Kelly, S., and Sanderson, W. S.: Comparison of various tissue cultures for the isolation of enteroviruses. Amer. J. Pub. Health, 1962, **52:**455–459.
77. Kilbourne, E. D.: The Coxsackie viruses and human disease. Amer. J. Med. Sci., 1952, **224:**93–102.
78. Kochs, H.: Live poliovirus vaccination in West Berlin. Amer. J. Pub. Health, 1962, **52:**611–615.
79. Koprowski, H., Jervis, G. A., and Norton, T. W.: Immune responses in human volunteers upon oral administration of a rodent-adapted strain of poliomyelitis virus. Amer. J. Hyg., 1952, **55:**108–126.
80. Korns, R. F., Albrecht, R. M., and Locke, F. B.: The association of parenteral injections with poliomyelitis. Amer. J. Pub. Health, 1952, **42:**153–169.
81. Krugman, S., et al.: Infectious hepatitis. Detection of virus during the incubation period and in clinically inapparent infections. New England J. Med., 1959, **261:**729–734.
82. Langmuir, A. D.: Progress in conquest of paralytic poliomyelitis. J. Amer. Med. Assn., 1959, **171:**271–273.
83. Langmuir, A. D., Nathanson, N., and Hall, W. J.: Surveillance of poliomyelitis in the United States in 1955. Amer. J. Pub. Health, 1956, **46:**75–88.
84. Larin, N. M.: Studies on the agent of canine virus hepatitis (Rubarth's disease). I. Complement-fixation and precipitin tests. J. Hyg., 1951, **49:**410–426.
85. Le Bouvier, G. L.: Poliovirus D and C antigens; their differentiation and measurement by precipitation in agar. Brit. J. Exptl. Path., 1959, **40:**452–463.
86. Le Bouvier, G. L.: The D→C change in poliovirus particles. Brit. J. Exptl. Path., 1959, **40:**605–620.
87. Ledinko, N., and Melnick, J. L.: Poliomyelitis viruses in tissue culture. III. Susceptibility of testicular tissue from immune monkeys. Proc. Soc. Exptl. Biol. Med., 1952, **81:**144–148.
88. Lepow, M. L., Robbins, F. C., and Woods, W. A.: Influence of vaccination with formalin inactivated vaccine upon gastrointestinal infection with polioviruses. Amer. J. Pub. Health, 1960, **50:**531–542.
89. Mansi, W.: The value of the complement fixation test in the study of canine distemper complex and Rubarth's disease. J. Comp. Path. Therap., 1955, **65:**291–308.
90. Mayer, M. M., et al.: The purification of poliomyelitis virus as studied by complement fixation. J. Immunol., 1957, **78:**435–455.
91. McLean, D. M., and Melnick, J. L.: Association with mouse pathogenic strain of ECHO virus type 9 with aseptic meningitis. Proc. Soc. Exptl. Biol. Med., 1957, **94:**656–660.
92. McLean, I. W., and Taylor, A. R.: Experiences in the production of poliomyelitis vaccines. Progress Med. Virol., 1958, **1:**122–164.
93. Melnick, J. L.: Poliomyelitis and poliomyelitis-like viruses of man and animals. Ann. Rev. Microbiol., 1951, **5:**309–332.
94. Melnick, J. L.: The Coxsackie group of viruses. Ann. N. Y. Acad. Sci., 1953, **56:**587–595.
95. Melnick, J. L.: Poliomyelitis. Adv. Virus Res., 1953, **1:**229–275.
96. Melnick, J. L.: Advances in the study of enteroviruses. Progress Med. Virol., 1958, **1:** 58–105.
97. Miles, J. A. R.: Changing views of the epidemiology of poliomyelitis. New Zealand Med. J., 1957, **56:**615–622.
98. Miles, J. A. R., Parry, H. B., Larin, N. M., and Platt, H.: Cultivation of canine hepatitis virus in embryonated hen's eggs and its subsequent transmission to dogs. Nature, 1951, **168:**699–700.
99. Mosley, J. W.: Water-borne infectious hepatitis. New England J. Med., 1959, **261:**703–708; 748–753.
100. Mosley, J. W.: Multiplication and cytopathogenicity of mouse hepatitis virus in mouse cell cultures. Proc. Soc. Exptl. Biol. Med., 1961, **108:**524–529.
101. Müller, F.: Die Gruppe der ECHO-viren. Eigenschaften, Pathogenität und Stellung in der Humanbiologie. Ergebn. Mikrobiol. Immunitätsf., 1961, **34:**275–362.

102. O'Malley, J. P., Meyer, H. M., and Smadel, J. E.: Antibody in hepatitis patients against a newly isolated virus. Proc. Soc. Exptl. Biol. Med., 1961, **108:**200–205.
103. Ormsbee, R. A., and Melnick, J. L.: Biologic and serologic characteristics of ECHO viruses from West Virginia. J. Immunol., 1957, **79:**384–392.
104. Paffenbarger, R. S., Jr., and Wilson, V. O.: Previous tonsillectomy and current pregnancy as they affect risk of poliomyelitis attack. Ann. N. Y. Acad. Sci., 1955, **61:** 856–868.
105. Pagano, J. S., et al.: Routine immunization with orally administered attenuated poliovirus. A study of 850 children in an American city. J. Amer. Med. Assn., 1960, **173:** 1883–1889.
106. Pagano, J. S., Plotkin, S. A., and Koprowski, H.: Variation of response in early life to vaccination with living attenuated poliovirus and lack of immunologic tolerance. Lancet, 1960, **ii:**1224–1226.
107. Paul, J. R.: Endemic and epidemic trends of poliomyelitis in Central and South America. Bull. W. H. O., 1958, **19:**747–758.
108. Paul, J. R.: Status of vaccination against poliomyelitis, with particular reference to oral vaccination. New England J. Med., 1961, **264:**651–658.
109. Payne, A. M.-M.: Oral immunization against poliomyelitis. Bull. W. H. O., 1960, **23:**695–703.
110. Peizer, L. R., Mandel, B., and Weissman, D.: An improved method for rapid laboratory diagnosis of poliomyelitis. Proc. Soc. Exptl. Biol. Med., 1961, **106:**772–776.
111. Pollard, M.: Comparative studies on viral hepatitis in animals and in man. Ann. N. Y. Acad. Sci., 1958, **70:**383–390.
112. Poskanzer, D. C., and Beadenkopf, W. G.: Waterborne infectious hepatitis epidemic from a chlorinated municipal supply. Pub. Health Repts., 1961, **76:**745–751.
113. Primavesi, K. A.: Der Virusnachweis bei der Hepatitis epidemica. Beiträge z. Hyg. u. Epidemiol., 1961, No. 14, pp. 1–72.
114. Quaade, F.: The influence of Salk's vaccine on the course of acute anterior poliomyelitis. Acta Med. Scand., 1960, **167:**427–430.
115. Quersin-Thiry, L., Nihaul, E., and Dekking, F.: ECHO virus type 9 as a cause of epidemic meningitis. Science, 1957, **125:**744–745.
116. Reploh, H., and Primavesi, K. A.: Epidemiologische Beobachtungen über eine Häufung von Erkrankungen an Hepatitis epidemica. Arch. f. Hyg. u. Bakt., 1960, **144:**287–307.
117. Report (Committee on Enteroviruses): The enteroviruses. Amer. J. Pub. Health, 1957, **47:**1556–1566.
118. Report: Expert Committee on Poliomyelitis. Third report. Washington, 13–16 June 1960. W. H. O., Tech. Rept. Ser., No. 203. World Health Organization, Palais des Nations, Geneva. 1960.
119. Report: Trial of living attenuated poliovirus vaccine. A report of the Public Health Laboratory Service to the Poliomyelitis Vaccines Committee of the Medical Research Council. Brit. Med. J., 1961, **ii:** 1937–1044.
120. Report: Hepatitis possibly transmitted by chimpanzees. Morbid. Mortal. Weekly Rept., 1961, **10:**No. 20.
121. Rightsel, W. A., et al.: Status report on tissue-culture cultivated hepatitis virus. Part I. Virology laboratory studies. J. Amer. Med. Assn., 1961, **177:**671–678.
122. Roca-García, M., and Jervis, G. A.: Experimentally produced poliomyelitis variant in chick embryo. Ann. N. Y. Acad. Sci., 1955, **61:**911–923.
123. Roizman, B., Mayer, M. M., and Rapp, H. J.: Immunochemical studies of poliovirus. III. Further studies on the immunologic and physical properties of poliovirus particles produced in tissue culture. J. Immunol., 1958, **81:**419–425.
124. Rosen, L.: Serologic grouping of reoviruses by hemagglutination-inhibition. Amer. J. Hyg., 1960, **71:**242–249.
125. Rosen, L., et al.: Observations on a newly recognized virus (Abney) of the reovirus family. Amer. J. Hyg., 1960, **71:**258–265.
126. Rosen, L., and Abinanti, F. R.: Natural and experimental infection of cattle with human types of reoviruses. Amer. J. Hyg., 1960, **71:** 250–257.
127. Rubin, H., et al,: Epidemic infection with Coxsackie virus group B type 5. I. Clinical and epidemiologic aspects. New England J. Med., 1958, **258:**255–263.
128. Ryder, D. E., Doane, F. W., Zbitnew, A., and Rhodes, A. J.: Report of an outbreak of Bornholm disease, with isolation of Coxsackie B5 virus: Toronto, 1958. Canadian J. Pub. Health, 1959, **50:**265–269.
129. Sabin, A. B.: Pathogenesis of poliomyelitis. Science, 1956, **123:**1151–1157.
130. Sabin, A. B.: Present position of immunization against poliomyelitis with live virus vaccines. Brit. Med. J., 1959, **i:**663–680.
131. Sabin, A. B.: Status of field trials with an orally administered live attenuated poliovirus vaccine. J. Amer. Med. Assn., 1959, **171:** 863–868.
132. Sabin, A. B.: Reoviruses. A new group of respiratory and enteric viruses formerly classified as ECHO type 10 is described. Science, 1959, **130:**1387–1389.
133. Sabin, A. B.: Eradication of poliomyelitis. Ann. Intern. Med., 1961, **55:**353–357.
134. Sabin, A. B., et al.: Community-wide use of oral poliovirus vaccine. Effectiveness of the Cincinnati program. Amer. J. Dis. Children, 1961, **101:**646–667.
135. Sabin, A. B., Hennessen, W. A., and Winsser, J.: Studies on variants of poliomyelitis virus. I. Experimental segregation and properties of avirulent variants of three immunologic types. J. Exptl. Med., 1954, **99:**551–576.
136. Salk, J. E.: Considerations in the preparation and use of poliomyelitis virus vaccine. J. Amer. Med. Assn., 1955, **158:**1239–1248.
137. Sanford, J. P., and Sulkin, S. E.: The clinical spectrum of ECHO-virus infection. New England J. Med., 1959, **261:**1113–1122.
138. Schaeffer, M., Robinson, R. Q., and Fodor, A. R.: New viruses and virus diseases of man. Ann. Rev. Microbiol., 1959, **13:**335–348.
139. Schaffer, F. L.: Interaction of highly purified poliovirus with formaldehyde. Ann. N. Y. Acad. Sci., 1960, **83:**564–577.
140. Schaffer, F. L., and Schwerdt, C. E.: Purification and properties of poliovirus. Adv. Virus Res., 1959, **6:**150–204.

141. Schindler, R., and Mohr, W.: Über eine Infektion mit dem Virus der Hepatitis contagiosa canis beim Menschen. Deut. med. Woch., 1959, **84:**2080–2084; 2104.
142. Schmidt, N. J., Fox, V. L., and Lennette, E. H.: Immunologic identification of Coxsackie A21 virus with Coe virus. Proc. Soc. Exptl. Biol. Med., 1961, **107:**63–65.
143. Schwerdt, C. E., and Schaffer, F. L.: Some physical and chemical properties of purified poliomyelitis virus preparations. Ann. N. Y. Acad. Sci., 1955, **61:**740–753.
144. Scott, T. F. McN.: Clinical syndromes associated with entero virus and reo virus infections. Adv. Virus Res., 1961, **8:**166–199.
145. Shelokov, A., Habel, K., and McKinstry, D. W.: Relation of poliomyelitis virus types to clinical disease and geographic distribution: Preliminary report. Ann. N. Y. Acad. Sci., 1955, **61:**998–1004.
146. Smorodintsev, A. A., et al.: Results of a study of the reactogenic and immunogenic properties of live anti-poliomyelitis vaccine. Bull. W. H. O., 1959, **20:**1053–1074.
147. Smorodintsev, A. A.: New data on live vaccines against poliomyelitis, mumps and measles. Progress Med. Virol., 1961, **3:**245–310.
148. Staff of Poliomyelitis Surveillance Unit: Poliomyelitis in the United States in 1956. Pub. Health Repts., 1957, **72:**381–392.
149. Symposium: Second international conference on live poliovirus vaccines (Washington, 6–10 June 1960). Scientific Publication No. 50. Pan American Sanitary Bureau, Regional Office World Health Organization, Washington. 1960.
150. Syverton, J. T., et al.: Outbreak of aseptic meningitis caused by Coxsackie B5 virus. Laboratory, clinical and epidemiologic study. J. Amer. Med. Assn., 1957, **164:**2015–2019.
151. Vainio, T.: Studies on murine hepatitis virus (MVH3) in vitro. Proc. Soc. Exptl. Biol. Med., 1961, **107:**326–331.
152. Vogt, M., Dulbecco, R., and Wenner, H. A.: Mutants of poliomyelitis viruses with reduced efficiency of plating in acid medium and reduced neuropathogenicity. Virology, 1957, **4:**141–155.
153. Voroshilova, M. K., and Chumakov, M. P.: Poliomyelitis-like properties of AB-IV-Coxsackie A7 group of viruses. Progress Med. Virol., 1959, **2:**106–170.
154. Ward, R., et al.: Infectious hepatitis. Studies of its natural history and prevention. New England J. Med., 1958, **258:**407–416.
155. Wenner, H. A., Archetti, I., and Dubes, G. R.: Antigenic variations among type 1 polioviruses. A study of 16 wild-type strains and 5 variants. Amer. J. Hyg., 1959, **70:**66–90.
156. Wenner, H. A., Kamitsuka, P., Lenahan, M., and Melnick, J. L.: A comparative study of type 2 poliomyelitis viruses. II. Antigenic differences relating to 18 type 2 strains. J. Immunol., 1956, **77:**220–231.

Chapter 40

THE VIRUSES OF ARTHROPOD-BORNE DISEASE AND OTHER NEUROTROPIC VIRUSES

THERE is a large group of viruses[28] which occur primarily in reservoirs of infection in lower animals, including birds. Many are transmitted to the vertebrate host by arthropod vectors, and infection of man is often peripheral to the life history of the virus.[143, 198] Many of them are neurotropic in that the symptoms of the human disease may be referable to involvement of the central nervous system, or the affinity for nerve tissue may be demonstrable in experimental infections. All are pathogenic for the adult or suckling mouse, and many have been cultivated in the embryonated egg and in various forms of tissue culture.[84] The group includes the virus of yellow fever, the viruses of the so-called arthropod-borne encephalitides and of other arthropod-transmitted disease, together with neurotropic viruses such as those of rabies and lymphocytic choriomeningitis.

YELLOW FEVER[194]

Yellow fever is an acute disease, occurring in epidemic and endemic form, which was first observed in Central America in the middle of the seventeenth century. It is uncertain whether the original focus of infection was in Africa or the Western Hemisphere, but in the eighteenth and nineteenth centuries the disease was widely distributed in the Caribbean area, including the adjoining coasts of North, Central, and South America. It occurred in epidemic form in more northern cities in this country, including Philadelphia, New York, and Baltimore, and it is estimated that there were at least half a million cases of the disease in this country in the nineteenth century. It occurred in epidemic form in Spain in 1800 with 60,000 deaths, and in Rio de Janeiro with more than 20,000 deaths between 1851 and 1883, and there were almost 36,000 deaths from the disease in Havana during the same period.

The occurence of the disease in American military personnel during the Spanish-American war resulted in the classic studies of the Yellow Fever Commission of the United States Army under Dr. Walter Reed at the beginning of the

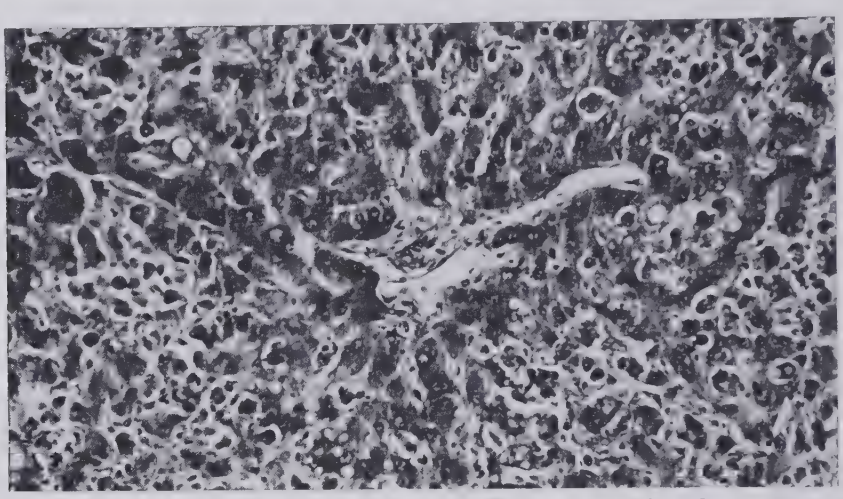


Figure 297. Section of liver from a human case of yellow fever. Midzonal necrosis is apparent. Hematoxylin and eosin; reduced from × 235. (Theiler.)

twentieth century, with evidence of mosquito transmission and the viral nature of the infectious agent. Later work appeared to suggest that the disease is a leptospirosis, but in the late 1920's its viral etiology was firmly established.[19, 151]

The virus, shown to be filterable by the work of the Commission, is one of the smaller viruses, with a diameter of 30 mμ, and it is readily filterable through the usual bacteria-proof filters. It is relatively labile, is readily inactivated by heating and by the usual disinfectants, but is preserved in 50 per cent glycerol and in lyophilized form.

Disease in Man. The disease is transmitted to man by the bite of infected mosquitos, and the incubation period was found to be 1 to 3 days in early experimental infections. The onset is sudden with headache, backache, and rigor, and the temperature rises rapidly to reach a maximum within 48 hours. In the first, or congestive, stage of the disease there is nausea and vomiting, the face is flushed, and a tendency to hemorrhage becomes apparent. After 3 or 4 days the temperature falls, then rises again, and the second stage of the disease is characterized by venous stasis and a marked tendency to hemorrhage; prostration occurs, jaundice appears but is not marked, and the kidneys are involved and there is albuminuria. Death occurs by the sixth or seventh day and seldom after the tenth day, or recovery is rapid and uneventful. Many infections are mild or almost symptomless, and the fatality rate is not known with precision; it is estimated to be about 5 per cent.

The disease is one of the hematopoietic system, there is little inflammatory reaction, and the tissue changes are degenerative in nature. These occur in the liver, kidney, and heart, and there is evidence of hemorrhage. The characteristic, and pathognomonic, lesion is a hyaline type of necrosis in the midzone of the liver, and the affected cells are known as Councilman bodies. Fatty degeneration occurs in the kidney tubules but is not distinctive, the spleen is hyperemic and shows degenerative changes, and degenerative changes are found in the heart.

Experimental Infections. The human disease is closely reproduced in the rhesus monkey,[12, 13, 14] and a number of species of monkeys are susceptible to yellow fever; in fact, natural infection appears to be widespread among primates in certain areas (see below). The neurotropic tendencies of the virus are demonstrable in the monkey by intracerebral inoculation, and simultaneous protection of the viscera by antiserum, to produce an encephalitis. The rabbit and rat are resistant to experimental in-

fection, but encephalitis is produced in the guinea pig on intracerebral inoculation. The infant mouse is susceptible to parenteral inoculation by any route; the adult mouse is susceptible to intracerebral inoculation but is seldom infected by intraperitoneal inoculation. Some strains of mice are considerably more susceptible than others. Passage by intracerebral inoculation in the mouse results in adaptation to the mouse brain, and such strains lose their viscerotropic tendencies and become less virulent for the monkey.

Tissue culture. Neurotropic virus is cultivable without difficulty in the Maitland type of tissue culture of chick embryo which includes nerve tissue, but the unadapted pantropic strains may be difficult to grow. The Asibi strain of yellow fever virus was grown first in mouse embryo tissue culture, then chick embryo tissue culture, and after prolonged passage a variant appeared which had lost both viscerotropic and neurotropic tendencies. This variant is the 17D strain, now widely used as an immunizing agent.

This strain will form plaques in chick embryo cell monolayers, and the system may be used to titrate antibody.[79, 154] It grows in HeLa and KB cell cultures with a cytopathic effect characterized by rounding up of the cells and detachment from the glass.[71] In human embryonic intestine, human amnion or Chang's liver cell culture the virus is demonstrable by the fluorescent antibody technique as a perinuclear fluorescence.[40]

Epidemiology. Yellow fever occurs in animal reservoirs of infection and in infected mosquitoes and is transmitted to the vertebrate host by the insect vector. The epidemiological character of the disease is determined by the species and habitat of both vertebrate and invertebrate hosts and occurs in two epidemiological types, the one urban yellow fever, and the other jungle yellow fever.

Urban yellow fever. It was established at the turn of the century that the disease is transmitted to man by *Aedes aegypti,* and in the urban type of disease the life history of the virus consists of a man-mosquito-man cycle in which the mosquito is infected by man, requires 10 to 15 days at tropical temperatures to become infectious, and remains in-

Figure 298. The geographical distribution of endemic yellow fever. Adapted from various sources.

fected for life. This mosquito is domestic, living largely in and around human habitation, the foci of infection are in urban areas, and the disease is one of communities. Since man-to-man infection does not occur under natural conditions, control of the vector in urban areas allows control of the human disease. Control programs directed toward this end have been highly successful, and the disease appeared to have been substantially eradicated in the Western Hemisphere by the middle 1920's.

By the late 1920's yellow fever appeared in small communities and rural areas in South America, and it was apparent that the man-mosquito-man cycle may persist in areas in which there are small groups of people in frequent communication with each other. Control consists of extension of mosquito control measures to such small communities and even to rural areas.

Jungle yellow fever. In the early 1930's yellow fever occurred in Brazil in the absence of *A. aegypti,* and the infection has been found to persist in reservoirs of infection in lower animals and to be transmitted by mosquitoes other than *A. aegypti.* This is sylvan or jungle yellow fever in which human infection is peripheral to the life history of the virus. The human disease is found in man coming in contact with forests or the edge of forests and tends to occur in adult males whose work takes them to such infected areas. This is in contrast with urban yellow fever in which all ages and both sexes are exposed to equal risk. Infection of man, followed by infection of *A. aegypti* may, of course, initiate the man-mosquito-man transmission cycle characteristic of urban yellow fever. The endemic foci of jungle yellow fever are in South America, Africa, and Central America, and differ somewhat from one to another of these areas.

South America. The area of endemic infection in South America, as indicated by the occurrence of antibody in young children as well as adults to show the recent or present occurrence of the disease, covers much of the tropical zone, including the Amazon and Orinoco regions, Colombia, the Guianas, and Panama. Within this area forest monkeys appear to be the most important vertebrate host, although in some areas where monkeys are few, marsupials such as the opposum show serological evidence of infection. Some species of monkeys, especially the howler monkey, Alouatta, suffer from epizootics of the disease which are fatal to large numbers of the animals. On the basis of the presence of antibody in the serum of wild caught specimens, the principal genera serving as vertebrate hosts seem to be Alouatta, Cebus, and Ateles, and others may be involved also.

Yellow fever virus has been isolated from forest mosquitoes, *Haemagogus capricornii, H. spegazzini,* and the subspecies *H. spegazzini falco.* Other species of the same genus, and one of the genus Trichoprosopon, transmit the disease experimentally and may be regarded as possible vectors. Species of Haemagogus are found over the entire area in South America in which the infection is endemic and are no doubt an important element in the transmission cycle. They are "forest-canopy," tree-hole breeding mosquitoes and are found in the tops of trees, rarely coming to the ground except at the edge of the forest.

Africa.[197] In Africa *A. aegypti* is widely distributed, and both the urban and jungle types of yellow fever occur. As in South America, serological evidence indicates that the vertebrate host in the latter form of the disease is forest primates of many species, including lemurs. *A. africanus* is found in many parts of central Africa and is known to be an important factor in the maintenance of yellow fever virus in the forests of Uganda. Like Haemagogus in South America, it is an arboreal mosquito, and it readily bites monkeys, but does not attack man. *A. simpsoni,* which bites both monkeys and man, is also present, and is thought to be the mosquito responsible for transmission of the infection from monkeys to man at the edge of the forest.

Central America.[26, 211] The available

evidence shows that the jungle type of yellow fever has been moving north. In 1954, after 25 years with no record of yellow fever, cases of the disease occurred in man in Port-of-Spain, Trinidad. Mortality in monkeys was high during that year, and yellow fever virus was isolated from sick monkeys. The virus was also recovered from Haemagogus mosquitoes, and some of the cases of the disease in man were probably acquired from *A. aegypti* which had not been kept under control, *i.e.*, the urban type of the disease. Similarly, in 1948 cases of yellow fever were reported in eastern Panama for the first time since 1905. It subsequently moved as a wave in a general northwesterly direction into central and western Panama, Costa Rica, and Nicaragua, and reached Honduras in 1954. In early 1956 yellow fever appeared in monkeys in Guatemala, and the virus was isolated from two species of mosquitoes, *H. equinis* and *Sabethes chloropterus*. The virus has also been recovered from other Central American mosquitoes, including *H. spegazzini falco, H. lucifer,* and an anopheline, but their status as vectors is uncertain.

In contrast to the situation in South America where Cebus monkeys are in the majority and show little mortality from naturally-acquired yellow fever, the howler (Alouatta) and spider (Ateles) monkeys are predominant in Central America and show a high mortality from yellow fever. In fact, the death of monkeys, amounting in some areas to virtual extermination, has been one indicator of the progress of the infection through Central America.

There is considerable concern[18, 48, 92] over this northward extension of the disease, for susceptible monkeys and mosquitoes are present in southern parts of Mexico, and there appears to be no geographical or climatic barrier to the spread of the disease. *A. aegypti* occurs northward up both coasts of Mexico and extends into the southern United States, and the occurrence of the urban form of yellow fever in those areas is a possibility.

Immunity. Recovery from yellow fever results in a solid immunity, often said to persist for life, and strains of the virus, even from widely separated areas, are of a single antigenic type. The antibody response is demonstrable as HI, complement-fixing and neutralizing antibody, and the three are not identical. The complement-fixing antibody appears later than neutralizing or HI antibody during infection and may not occur to significant titer in mild infections. It disappears within a few months, and is usually not formed in response to prophylactic inoculation with the 17D strain of virus, and therefore has some value in detecting recent infections.[203]

The neutralizing antibody appears quite early in the disease, often by the fifth day, and persists more or less indefinitely. The titration of neutralizing antibody is used for serological surveys for the prevalence of infection. It has been carried out in various ways, and at the present time the method most commonly used consists of mixing varying dilutions of the serum with a standard amount of virus and testing for residual activity by intracerebral inoculation of adult mice or intraperitoneal inoculation of infant mice.

Prophylaxis. While vector control suffices to control urban yellow fever, it is ineffective in the control of the jungle type of the disease, and in this and certain other circumstances active prophylactic immunization is required. It was early apparent that inactivated virus does not induce an effective immune response,[39] and living attenuated virus is used. Two kinds of virus are used for this purpose, one a mouse-adapted neurotropic strain which is used largely by the French in Africa, and the other the 17D strain described above.

The former is employed as a lyophilized preparation which is suspended in a solution of gum arabic just before use and applied by scarification. As used by the French workers in Africa, it is often combined with vaccinia virus. Vaccine is prepared from the 17D strain as lyophilized chick embryo tissue, recon-

stituted in saline, and inoculated subcutaneously in doses containing 500 mouse MLD. The incidence of untoward reactions is low, perhaps 5 per cent, and such reactions are usually mild. The neurotropic strain appears to give a somewhat better immune response than the 17D strain as measured by neutralizing antibody titer, and in either case the immunity so produced is effective for considerable periods of time, at least 5 to 6 years.[150]

THE ARTHROPOD-BORNE ENCEPHALITIS AND RELATED VIRUSES[11, 106, 157, 171]

While "sleeping sickness," which may be of bacterial or protozoan etiology, has long been known to occur as sporadic cases and in epidemic form, sometimes associated with other disease, the first encephalitis of probable viral etiology to be described with sufficient accuracy for tentative identification was encephalitis lethargica or von Economo's disease. This disease occurred in Rumania in 1915, in France in 1916, was described by von Economo in Vienna in 1917, and apparently spread to this country in 1918. Epidemics occurred in various parts of the world until 1926 but not thereafter. The disease was not of bacterial etiology, but no causative virus was isolated, and the disease has only an uncertain clinical identity. If it was a definite entity, it is not known why it failed to occur after 1926.

Study of the viral encephalitides began in the early 1930's with the isolation of a variety of viruses causing, or potentially capable of causing, encephalitis. The neurotropic character of some of these is marked, but in others it is latent, so that involvement of the central nervous system is relatively rare in the naturally-occurring disease and may be demonstrable only under experimental conditions as in the case of yellow fever virus described above. Certain of these viruses tend to form a group, related to some degree by antigenic character as well as by spread by arthropod vectors and their occurrence as natural infections of lower animals, and are set apart from other viruses, such as those of herpes simplex, the psittacosis group, mumps, and measles, which may at times infect the central nervous system.

EQUINE ENCEPHALOMYELITIS VIRUSES

Encephalomyelitis of horses and mules occurring as summer epizootics in North America has been known for many years.[192] A virus was recovered by Meyer and his his associates from the brains of horses during an epidemic of the disease in California in 1930 and was found in the blood and central nervous system of human cases of the disease by Howitt in 1938. A similar equine disease occurs in the eastern part of the United States and Canada, and a virus was isolated from infected animals in 1933 by Ten-Broeck and Merrill during an epizootic occurring in the central Atlantic Coast states, and from the central nervous system of human cases of the disease in 1938 by Fothergill and his co-workers. A third kind of encephalomyelitis of horses and mules occurs in the northern portion of South America, and is known as Venezuelan equine encephalomyelitis. The causative virus was recovered from infected animals in 1938, laboratory infections in man were reported in 1943, and naturally occurring human disease in the following year. The three viruses are similar in many respects but are distinct antigenically and differ in other ways. The North American viruses have

been intensively studied and are somewhat better known than the Venezuelan virus.

Western Equine Encephalomyelitis

The western equine encephalomyelitis or WEE, virus is about 50 mμ in diameter as indicated by sedimentation rates, although filtration experiments give a smaller value, 20 to 30 mμ. Chemical analysis of purified preparations has shown about 50 per cent lipid, 4 per cent carbohydrate, and the remainder the ribose type of nucleoprotein, and the virus appears to be a lipid-nucleoprotein complex. It is relatively stable to heat, for 10 minutes at 60° C., and to disinfectants such as ether, phenol, mercuric chloride, and phenyl mercuric borate, and is inactivated by 0.4 per cent formalin after 2 to 4 days. The last accounts for accidental infections which have occurred following the inoculation of formalin-inactivated experimental vaccines. It is readily cultivable in the embryonated egg and chick embryo by all routes of inoculation to produce a characteristically rapidly fatal infection of the embryo with generalized hemorrhage, thrombosis and necrosis, and death within 24 hours. It also grows in a variety of tissue cultures,[21, 103] and in both these and egg cultures large amounts of virus are produced.

Disease in Man. The incubation period is from less than 1 to as long as 3 weeks, and the human disease varies from an almost symptomless or abortive form to an acute disease in which the patient becomes comatose within 24 hours. The disease is essentially a meningo-encephalitis with rarely any involvement of the medulla or spinal cord. In the typical case the prodromal signs and symptoms may include headache, drowsiness, and fever. Symptoms of central nervous system involvement may also include tremor, convulsions, mental confusion, and amnesia, but paralysis is not common, occurring in perhaps 15 per cent of cases. The acute phase of the disease lasts 7 to 10 days; recovery is usually uneventful and complete. In California in 1952, a peak year, 729 cases of encephalitis occurred of which almost half were WEE infections, and the case fatality rate in that outbreak was 7 per cent. Serological evidence indicates, however, that many infections with this virus never result in clinical encephalitis, and many others are subclinical.

Experimental Infections. The experimental animal of choice is the mouse which may be infected by the intranasal, intraperitoneal, or intracerebral route. Following intraperitoneal inoculation, the virus appears in the blood, infects the nasal mucosa, and reaches the central nervous system via the olfactory nerves. Within a few days the animals show signs of meningo-encephalitis, such as spastic muscular contractions and paralysis, followed by prostration and death, and the disease is similar to that in man. The infection of other animals, including rabbits, guinea pigs, and a number of birds, by the intracutaneous route results in an inapparent infection with viremia to give the kind of infection that probably occurs in animal reservoirs of the virus.

Immunity. Both complement-fixing and neutralizing antibodies are produced as a result of infection and are associated with a solid immunity. Antibody appears early in the infection, usually within a week of onset, and a rise in titer between paired serums is diagnostic. The complement-fixing antibody usually disappears within a year, but the neutralizing antibody persists for longer periods and is the one titrated in serological surveys. Experimental animals may be immunized by the inoculation of formalin-inactivated mouse brain antigen, and in such animals the immunity to challenge inoculation is correlated with the titer of neutralizing antibody in the serum. Mice may be effectively protected against both intraperitoneal and intracerebral inoculation, but an abortive infection is produced in the immunized guinea pig on intracerebral challenge. Mouse brain and chick em-

bryo vaccines have not been particularly successful in the immunization of vated vaccine.[128]

Naturally-acquired infection occurs in marily an infection of wild birds which is transmitted by mosquitoes, and, in the natural history of the virus, infection of horses and man is incidental. The infection occurs west of the Mississippi River, but both serological evidence and isolation of the virus have shown that it is also present in some areas along the Atlantic coast, including New England,[82] and on the Gulf coast.

Hosts. The virus has been recovered from about twenty species of wild birds and six species of mammals, and there is serological evidence of infection in more than seventy-five species of wild birds and most common domestic birds and mammals.[107] It has been shown experimentally that the level of viremia is considerably higher in infected wild birds than in domestic birds or mammals, and it may be inferred that mosquitoes may be more readily infected by feeding on the former. While this and other evidence leaves little doubt that wild birds are the important natural reservoir of infection,[159] and that infection in mammals and domestic birds does not play a significant part in the transmission cycle, it is not clear which species of wild birds are of primary importance and which are only secondary. It has been found also that garter snakes (Thamnophis sp.) may be infected, by inoculation or by infected *C. tarsalis,* the infection remaining latent during winter hibernation, and then becoming activated in the spring to levels of viremia sufficient to infect mosquitoes.[59, 204, 206, 207]

Vectors. WEE virus has been isolated from twelve or more species of mosquitoes, from several species of bird mites, and from the assassin bug, Triatoma. Of these, it seems clear that *Culex tarsalis* is the primary vector of the infection, both in its natural transmission cycle and in the endemicity of the disease in man and horses in the western part of the country. The conclusion is based on detailed consideration of factors such as population density and blood-feeding habits as well as the consistent association of this mosquito with epidemics of the disease in mammals. WEE exists, however, in the absence of *C. tarsalis,* as in the endemic areas on the Atlantic and Gulf coasts, and has been isolated in those regions from species of Culex, Aedes, and Culiseta. These or other vectors are sufficient to maintain the infection at a relatively low endemic level, with transmission to both birds and mammals, but the relative unimportance of WEE in these areas is apparently due to the absence of an efficient vector such as *C. tarsalis* and possibly also to a degree of cross-immunity with the eastern virus which results in a lower level of viremia when WEE infection occurs in birds or mammals immune to the eastern virus.

Eastern Equine Encephalomyelitis

Eastern equine encephalomyelitis, or EEE, is a disease similar to WEE but differing in its geographical distribution, arthropod vectors, and host range. It occurs in Atlantic and Gulf coast states from New Hampshire to Texas and has been found as far west as Wisconsin in this country, in Canada, Mexico, the Caribbean area including parts of Central and South America, and in the Philippines.

The EEE virus is closely similar in size and chemical composition to the WEE virus and also produces rapidly fatal infections in the chick embryo and cytopathological changes in a variety of kinds of tissue cultures. Its pathogenicity for experimental animals parallels that of WEE virus, but it has a somewhat wider host range and is able to infect sheep, cats, and hedgehogs. A comparison[140] of tissue cultures and experimental animals has shown that chick embryo cells in tissue culture are the most sensitive of the tissue cultures and the suckling mouse the most susceptible experimental animal host. Chick embryo

cell culture of the virus has been used for the preparation of formalin-inactivated vaccine.[128]

Naturally-acquired infection occurs in both horses and man. In the former the disease is substantially identical with that produced by WEE virus, but infections in man are relatively more severe and are characterized by a higher case fatality rate and a tendency to occur in children. A total of fifty human cases of the disease occurred in Massachusetts in small outbreaks in 1938, 1955, and 1956. On analysis[51] of these cases it was shown that 46 per cent of the affected individuals were less than 3 years old and 68 per cent under 10 years of age. Thirty-four of the fifty cases were fatal, and recoveries were less satisfactory in younger survivors, with mental retardation, epilepsy, and paralysis of varying extent occurring as sequelae. Similarly, of thirteen cases of the human disease which occurred in the Dominican Republic in 1948–1949, only one person was over 8 years of age, and nine of the patients died.

Epidemiology.[34, 86, 104] As in WEE, the natural reservoir of infection of EEE is in wild birds, with infection in mammals and domestic birds playing only a minor part in the natural transmission cycle. Infection of man and the horse is incidental and occurs during periods of high prevalence of the infection.

Hosts. A wide variety of wild birds, more than forty species, have been found to show serological evidence of infection with EEE virus. In a general way, the smaller birds, including grackles, cardinals, and sparrows, show a higher level of viremia on experimental infection than do larger birds, such as herons and pheasants, and also tend to be more numerous. Further, the proportion of birds showing serum antibody is greater in migratory birds which winter south of the United States than in birds wintering in the north, suggesting a transmission cycle in Central or South America in birds found in this country in the summer.[108]

There is evidence of fluctuation in the prevalence of the infection as indicated by the proportion of successful virus isolations and of birds showing the presence of serum antibody. During periods of low prevalence the incidence of virus isolations is less than 1 per cent and the incidence of antibody 15 to 20 per cent in caught wild birds, and the birds involved include the catbird, cardinal, hermit thrush, and yellow-crowned night heron. During periods of high prevalence of infections, virus has been isolated from as much as 10 per cent of birds examined, and 50 per cent or more show the presence of antibody in the serum. At the same time the virus is found in additional species, such as the English sparrow and pigeon, and infection of horses and man may represent a similar spill-over of infection during periods of high prevalence.

It is of some interest that outbreaks of EEE have occurred on pheasant farms in Connecticut and in New Jersey. Presumably the infection is acquired initially from an arthropod vector, but direct bird-to-bird transmission occurs to give rise to epidemics of the disease. It is unlikely that caged pheasants play any significant part in the maintenance of the infection but may be considered as a sensitive indicator of the presence of the virus in the vicinity.[93, 99, 129, 170]

Vectors. EEE virus has been isolated from a number of species of mosquitoes, including *Culiseta melanura, Mansonia perturbans, Anopheles crucians, Culex salinarius, Aedes mitchellae,* and pooled Culicoides. *C. melanura,* in which the proportion of isolations has been relatively high, is a fresh-water swamp mosquito which bites birds freely. The distribution of this mosquito, and *A. mitchellae* and the saltmarsh species of Culicoides, coincides with the endemic areas of EEE virus infection. It is believed that *C. melanura* is one of the most important vectors in the maintenance of the natural transmission cycle, but it seldom bites man or the horse, and it is probable that other vectors are involved. *A. sollicitans,* for example, has been found to be an excellent experimental vector, but *C. pipiens* is not. It is possible also that, with a high infec-

tion rate in the horse, mechanical horse-to-horse transmission may occur as a consequence of interrupted feeding of various biting insects such as stable flies, deer flies, and mosquitoes, for such mechanical transmission is demonstrable experimentally.

Venezuelan Equine Encephalomyelitis

Venezuelan equine encephalomyelitis, or VEE, occurred as an epizootic in horses and mules in Colombia in 1935, and the causative virus was isolated during a similar epizootic in Venezuela in 1938. The disease has also been observed elsewhere in South America, *i.e.*, in Argentina, Ecuador, and Trinidad.

The infection in man usually results in a relatively mild, influenza-like disease with headache and fever, gastrointestinal disturbance, myalgia, and lethargy, with little or no indication of central nervous system involvement. Such cases occur as a result of naturally-acquired infection, as in Colombia in 1952[169] and have occurred repeatedly as a result of laboratory infection. The disease lasts for 3 to 5 days, occasionally longer in more severe cases. Fatal cases are rare, and in two observed in Trinidad the onset was sudden with signs of encephalitis, followed by coma and death. On the other hand, serological evidence indicates that symptomless or subclinical infections in man may be relatively common in endemic area. In man the virus is present in the blood and also in the nasopharnyx, but there is no evidence of man-to-man transmission of the respiratory infection.

VEE virus infects horses and mules but not cattle. It is less pathogenic for birds, and occasional birds, especially pigeons and doves, appear to be refractory to experimental infection. In contrast with WEE and EEE viruses, there is a high-level viremia in infected horses but only a low-level viremia in infected birds. It is probable that the reservoir of infection is mammalian rather than avian and that the disease is transmitted by an insect vector; *Mansonia titillans* transmits the infection under experimental conditions, but the vector of the

Figure 299. The geographical distribution of the western and eastern varieties of equine encephalomyelitis and of St. Louis encephalitis in the United States. The circles represent sporadic occurrence. Redrawn from Hess and Holder.

naturally-occurring disease is not known.

Little is known also of the physical or chemical character of VEE virus, but it is immunologically distinct from WEE and EEE viruses. The usual laboratory animals are readily infected by peripheral inoculation, producing a high level of viremia and encephalitis, and the most useful experimental animals have been the mouse and the guinea pig. The virus grows profusely in the chick embryo and embryonated egg and is cultivable in tissue cultures of human uterus, HeLa cells, and guinea pig and hamster kidney cells.[56, 103] Experimental animals may be immunized with formalin-inactivated chick embryo virus, and VEE vaccine has been combined with WEE and EEE vaccines for the immunization of laboratory personnel. A variant, avirulent by other than the intracerebral route, which is an effective immunizing agent under experimental conditions has been described.[83]

ST. LOUIS ENCEPHALITIS

The disease known as St. Louis encephalitis, or SLE, was first observed in eastern Illinois in 1932 where it was diagnosed as von Economo's disease, and in the following year it occurred in epidemic form in and around St. Louis with an attack rate of 1 per 1000 and a case fatality rate averaging about 20 per cent. The infection occurs in the central states of the midwest and over the entire western half of this country, coinciding with WEE in the latter respect, and has been found in birds and mosquitoes in Trinidad. SLE, WEE, and EEE together make up the most important arthropod-borne viral encephalitides in this country.

The SLE virus was isolated in 1933 by the intracerebral inoculation of monkeys. In many respects, including a size of 20 to 30 mμ, it is closely similar to the WEE and EEE viruses described above, and other encephalitis viruses such as Japanese B encephalitis virus (see below), but it is immunologically distinct and differs in its pathogenicity for various experimental animals. The virus is cultivable in chick embryo and mouse embryo tissue culture and in the yolk sac or chorioallantoic membrance of the embryonated egg. Pocks are not produced on the chorioallantoic membrane, but the infected membrane is edematous, with evidence of cell proliferation and focal necrosis. In contrast to the WEE and EEE viruses, SLE virus infection of the embryonated egg does not result in rapid death of the embryo.

The susceptibility of rhesus monkeys to intracerebral inoculation is somewhat irregular, only some strains producing an infection, and the virus tends to be lost on passage; the cebus monkey appears to be refractory to infection. Infant mice are the most susceptible of the usual experimental animals and may be infected by all routes of inoculation, and adult mice may be infected by intracerebral or intranasal inoculation. Only an inapparent infection is produced in rabbits, guinea pigs, and various birds, and adult rats, sheep, and ferrets are refractory to infection.

Disease in Man. The disease in man is closely similar to WEE, ranging in severity from an abortive type of infection with headache and fever to a severe disease with abrupt onset and sypmtoms including abdominal and muscular pain, sore throat, conjunctivitis, and signs of neurological disturbance such as ataxia, mental confusion, and occasionally a spastic type of paralysis. The incidence of the disease is relatively high in infants, least in older children, and rises again in the higher age groups. The fulminating type of disease tends to occur in infants, and in a considerable portion, 10 to 40 per cent, of recovered cases there may be evidence of damage to the central nervous system such as mental retardation and hydrocephalus. Recovery in the older groups is usually complete, with sequelae in 5 per cent or less of survivors, but the case fatality rate rises with age, and recovery is often protracted, to several months in severe cases. There is evidence which suggests that inapparent infection may be common.[20]

Complement-fixing and neutralizing antibodies appear in the serum by the end of the first week of the disease, and a rise in titer between paired serums is diagnostic. Neutralizing antibody, titrated in the mouse, persists for long periods, possibly for life, but complement-fixing antibody declines to insignificant levels within 2 to 3 years.

Epidemiology.[34, 86, 104] Unlike WEE, which occurs in the summer, SLE tends to occur in late summer and early fall. Following an outbreak in St. Louis in 1937, the disease appeared in sporadic form for several years, small urban and suburban outbreaks have occurred since 1954, and an epidemic of over 100 cases occurred in Louisville, Kentucky, in 1956, and one of a similar magnitude in St. Petersburg, Florida, in 1962.

Hosts. The reservoir of SLE virus infection, like that of WEE and EEE, is in birds, and the disease is transmitted by mosquitoes. The virus has been recovered from several species of birds, and serological evidence of infection has been found in fifty-five species of wild birds and a number of mammals, as well as in most domestic birds and mammals examined. The level of viremia is relatively low, but mosquitoes seem to be more readily infected with this virus than with others in which a high level of viremia is required for infection of the vector. The more important vertebrate hosts appear to be the small perching birds, such as finches and sparrows, and there is also reason to believe that chickens are important epidemic hosts for the virus.

Vectors. SLE contrasts with WEE with respect to arthropod vectors in that the mosquito susceptibility range tends to be reversed. WEE and EEE readily infect species of Aedes and Psorophora, but not Culex except *C. tarsalis,* while SLE is highly infectious for species of Culex but develops poorly in Aedes and Psorophora. These differences are reflected in the epidemiological pattern of the disease, and SLE occurs in two forms, the urban and rural types. The former, in which the disease is found in populated areas of the Midwest where *C. tarsalis* is scarce or absent, is transmitted to man by mosquitoes of the *C. pipiens* complex, including *C. pipiens, C. quinquefasciatus, C. molestus,* and possibly others. In western and rural areas, however, the primary vector appears to be *C. tarsalis.*

In the rural type of disease in the West, the transmission cycle includes wild, and possibly also domestic, birds as reservoirs of infection, maintained by transmission of the virus by *C. tarsalis,* with occasional infections occurring in man. In more densely populated areas in which *C. tarsalis* is seldom present, the infection is presumably introduced by wild birds and transmitted to local wild and domestic birds, and also to men, primarily by *C. pipiens* and *C. quinquefasciatus,* and outbreaks of the disease in man are associated with relatively dense populations of these mosquitoes.

JAPANESE B ENCEPHALITIS AND RELATED VIRUSES

Encephalitis of viral etiology occurs with some frequency in various parts of the Far East, and a related form of viral encephalitis has been found in Australia. A closely similar virus appears to be widespread in Africa associated with febrile disease but usually not with symptoms of encephalitis. These viruses are related to SLE and yellow fever viruses on the one hand, and to the Russian spring-summer encephalitis and related viruses on the other.

Japanese B Encephalitis[215]

Encephalitis has occurred in Japan in the late summer for many years and has assumed epidemic form from time to time. The disease has been called Japanese B encephalitis, or JBE, to distinguish it from von Economo's disease which was called Japanese A encephalitis in Japan. An epidemic of over 6000 cases with a 60 per cent case fatality rate occurred in

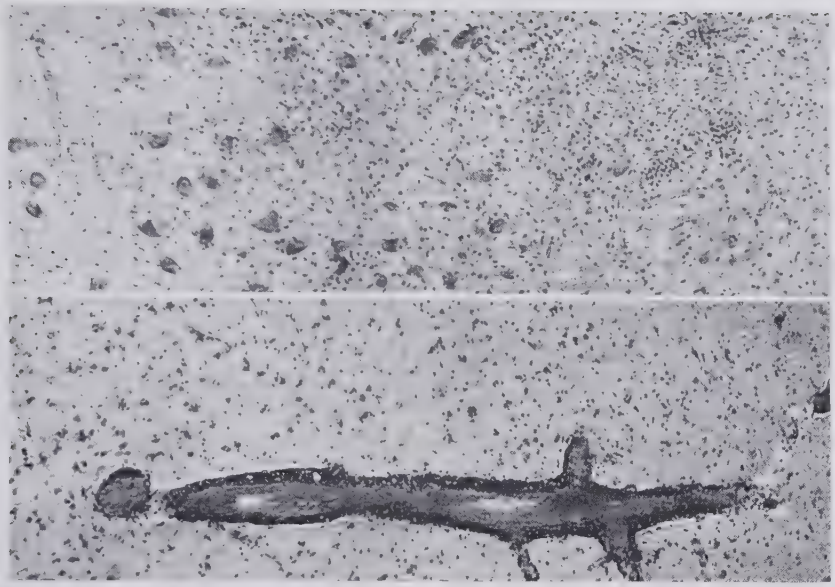


Figure 300. Pathological changes resulting from Japanese encephalitis infection. *Above,* substantia nigra from a human case; foci of mononuclear cell infiltration are present, and numerous ganglion cells are in a disintegrated state or have disappeared. Hematoxylin and eosin; × 90. *Below,* region of superior temporal gyrus from a human case; a vein at the corticomedullary junction is surrounded by a "cuff" of lymphocytes. (Haymaker and Sabin, Arch. Neurol. Psychiat.)

1924, and outbreaks occurred every summer to give, in the period 1924 to 1940, a total of approximately 27,000 cases of acute encephalitis. The infection has been found in many parts of the Far East, including China, Russia, Formosa, Thailand, Burma, Malaya, India, the Philippines, and portions of Indonesia, and an epidemic of more than 5500 cases with a 50 per cent case fatality rate occurred in Korea in 1949. Isolation of the virus was reported in 1936 by Japanese workers, Kasahara and his colleagues, and Taniguchi and his colleagues.

The virus is closely similar to those of the equine encephalitides and SLE in size and properties but has a relatively wider range of pathogenicity for experimental animals. The mouse, monkey, hamster, and young rat are susceptible in that order, the guinea pig is relatively resistant, and rabbits and chickens respond with viremia. Domestic animals, such as the horse, cow, sheep, and goat, may be infected experimentally, and show serological evidence of naturally-occurring infection in endemic areas, and epidemics of encephalitic disease in horses are often associated with epidemics of the disease in man. In the rhesus monkey an acute encephalomyelitis with relevant symptoms which simulates the human disease is produced following intracerebral or intranasal inoculation, and, in contrast with SLE virus, the infection may be carried in monkey passage. The virus is cultivable in the chick embryo and on the chorioallantoic membrane of the embryonated egg with death of the embryo within 72 hours. It will also grow on a variety of tissue cultures.[17, 120]

Disease in Man. The disease occurs in man as a symptomless or subclinical infection, an abortive type of disease in which fever and fleeting signs of central nervous system involvement occur, and as acute meningo-encephalomyelitis. In the last there is extensive cortical damage with focal perivascular infiltration, ganglion cell degeneration, and destruction of the Purkinje cells of the cerebellum, and there are lesions in the cord simulating those of poliomyelitis. The onset may be abrupt or insidious, and is characterized by fever, nausea, and disorientation. In the severe disease the

neurological symptoms are marked, and include spinal rigidity, tremors, convulsions, and spasticity. The acute phase of the disease may last as long as two weeks, but recovery is usually complete, and sequelae such as neurological or psychotic changes occur in less than 10 per cent of survivors.

The immune response is evident as the appearance of complement-fixing and neutralizing antibodies in the serum and occurs within 1 to 2 weeks after onset of the disease. The neutralizing antibody persists for a long time, and the associated immunity to the disease is thought to be relatively permanent. Complement-fixing antibody may appear in immunes following inoculation of vaccine that will not induce the formation of this antibody to significant titer in the normal individual. A formalin-inactivated chick embryo vaccine has been used for human immunization with suggestive results.[210] Another approach is that of primary inoculation with heterologous but related living virus, the West Nile virus (see below), followed by inoculation with inactivated vaccine of other viruses such as that of JBE.[94]

Epidemiology. Natural infection of mammalian hosts other than man with the virus occurs, as indicated by extensive outbreaks among horses and an infection of swine which results in abortion, stillbirth, or affected young, and other domestic animals show serological evidence of infection. Bats may be infected experimentally to give a mosquito-bat-mosquito cycle,[119] but its occurrence in nature is not known. Serum antibody has also been found in a number of species of Japanese birds, suggesting that there may be avian as well as mammalian reservoirs of infection.[77]

A number of species of culicine mosquitoes, including *C. tritaeniorhynchus* and *C. pipiens* var. *pallens*, can be infected with the virus, and can transmit the infection under experimental conditions. *C. tritaeniorhynchus* has been found to be naturally infected in Japan, and this and other evidence suggests that this mosquito may be one of the principal vectors of the disease there.[73]

Murray Valley Encephalitis

Encephalitis of viral etiology has occurred in Australia from time to time, and the virus appears to be a variant of the JBE virus. Epidemics of acute encephalitis occurred during the summer months between 1917 and 1926, in the first of which the disease occurred largely in small children, with a case fatality rate of 70 per cent, but subsequent epidemics were not as severe. In the severe form the disease was fulminating and resembled JBE in both symptoms and pathology. A virus was isolated from the central nervous system by inoculation of monkeys but unfortunately was lost before its characteristics could be determined, and the disease was called Australian X disease. It has not recurred since 1926.

In the summer of 1950–1951 an epidemic of encephalitis occurred in the Murray Valley in Victoria, Australia, in which forty cases were recognized and seventeen of these were fatal. A few cases occurred in 1956[5] and the disease seems to appear only sporadically. It has also been observed in New Guinea where serological surveys have suggested a patchy distribution of the infection.[6, 54] The disease is called Murray Valley encephalitis, but there is reason to believe that it represents a recrudescence of Australian X disease.

A virus was isolated on the chorioallantoic membrane of the embryonated egg, killing the embryo in 2 to 3 days, and adapted to infant mice in which it produced encephalitis, fatal in about a week, on intracerebral inoculation. The virus was found to be closely similar to, but not immunologically identical with, JBE virus.[184]

Complement-fixing and neutralizing antibodies, the latter demonstrable in the mouse, are produced in response to infection, and surveys in the epidemic area showed that about 5 per cent of persons with no history of the disease had significant titers of complement-fixing antibody. It was found in further studies that antibody is relatively widely distributed throughout eastern Australia,

not only in man, but also in horses and a number of species of wild birds.[144] There is circumstantial evidence which suggests that *C. annulirostris* transmits the disease.[135] The disease may, like SLE and the equine encephalitides, exist in a reservoir of infection in wild birds, maintained by mosquito, and possibly bird mite, transmission, which is transmitted to man and other mammals by mosquitoes.

West Nile Virus

The isolation of this virus from a native of Uganda with a mild febrile illness was reported by Smithburn in 1940; it was subsequently isolated in Egypt in 1951;[141] and outbreaks of the disease caused by it have been observed in Israel.[63, 131] In contrast to JBE, the disease is rarely fatal and usually does not take the form of encephalitis. The incubation period of the disease in Israel was 2 to 6 days, and it was characterized by an abrupt onset, fever, drowsiness, and severe frontal headache. A maculopapular rash may develop, more commonly in children, there may be abdominal pain with anorexia and nausea, and there is a general enlargement of the lymph nodes. The acute phase of the disease persists for less than a week, but convalescence may be protracted.

The virus is closely similar to the JBE virus and is of the same order of size and immunologically related to it and to SLE virus. It has been of particular interest in connection with active immunization to infection with closely related viruses such as JBE virus and others. Mice may be infected by the intracerebral route, and the infection is characterized by degenerative changes in the Purkinje cells of the cerebellum in both these animals and in monkeys. The virus grows on the chorioallantoic membrane of the embryonated egg and in a number of kinds of tissue cultures.

Serological evidence indicates that the infection is widespread in Africa, probably as inapparent infections, not only in Uganda and Egypt, but also in South Africa, the Belgian Congo, and the Sudan. As many as 70 per cent of persons examined in Egypt show significant antibody titer, and the disease has been studied extensively there.[201] The disease occurs largely during the summer and is sporadic rather than epidemic in form. The occurrence of outbreaks of the disease in Israel in 1951 and 1954 may have been due in part to relatively recent introduction of the virus.

The virus has been isolated from birds, pigeons, and the hooded crow, in Egypt, and there may be an avian reservoir of infection. Virus has also been found in *C. univittatus* and *C. anten-*

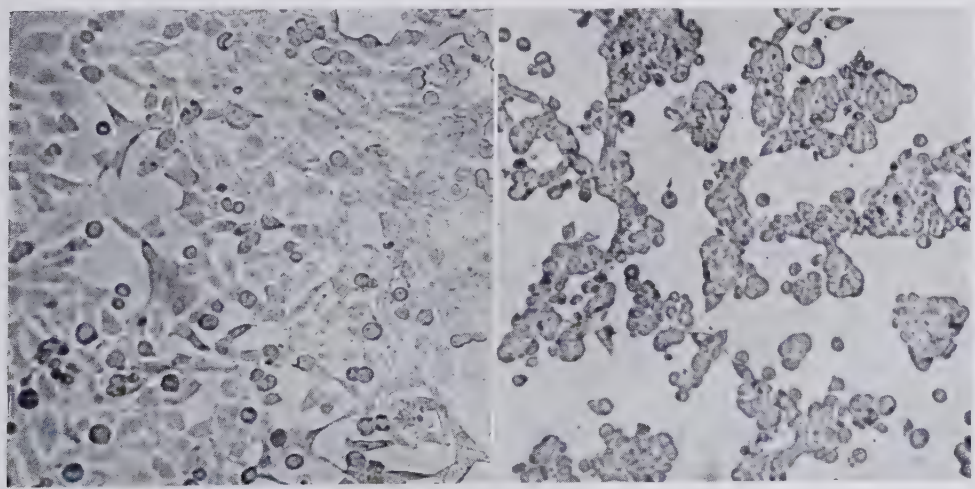


Figure 301. The cytopathogenesis of West Nile virus in HeLa cell culture. *Left,* uninoculated cell culture; *right,* 4 days after inoculation with virus.

natus, and *C. pipiens* and *A. aegypti* transmit the disease experimentally. *C. univittatus* is regarded as the most probable important vector of the infection.

DENGUE[80, 164, 166, 167]

Dengue, or breakbone fever, is an infectious disease, transmitted by mosquitoes, which is endemic, and sometimes epidemic, in tropical and subtropical climates; it may occasionally occur in temperate zones. Epidemics may be very large; it is estimated that there were between one and two million cases of the disease in the southern states in this country in the epidemic of 1922, more than a million cases occurred in Greece in 1927–1928, and a greater number in Japanese port cities from 1942 to 1945, with one-third to one-half the population of Osaka affected in 1944. It was first studied in the Philippines by medical officers of the United States Army who established the viral nature of the etiological agent and its transmission by *A. aegypti*.

The virus is similar in size to the yellow fever and encephalitis viruses described above, possibly slightly smaller, and its size is usually given as 12 to 25 mμ. In electron micrographs dumbbell-shaped particles are observed, but it is not established that these represent virus particles. The virus occurs as two immunological types, types 1 and 2, which are related by common antigens to one another, and also to certain other arthropod-borne viruses (see below).

Various monkeys, including species of Cynomologus, Ceropithecus, and Macacus, and chimpanzees may be infected with the production of antibody, but the infections are symptomless, and even inapparent infections are not produced in the usual experimental animals. Newborn mice, however, may be infected by intracerebral inoculation, with the development of immunity but no symptoms, or minor symptoms, of disease on primary isolation.

On passage the virus may become adapted to mice, giving high titers of virus in the brain, producing motor disturbance and partial flaccid paralysis with or without signs of encephalitis. Adult mice may be infected by intracerebral inoculation of well-adapted strains but are refractory to other routes of inoculation. A number of strains of both types of the virus have been so adapted to mice. Adapted virus may produce fatal paralytic disease in rhesus monkeys, simulating experimental poliomyelitis, following intracerebral inoculation.

Modification of the virus by adaptation also may result in loss of virulence for man, but not immunogenic potency, and modified virus may be used as an immunizing agent. It is not established that unmodified or modified virus can be grown in the embryonated egg. It has been reported that the virus is cultivable in monkey kidney tissue culture but grows slowly and with only irregular cytopathogenic effects.[89, 90, 91] In general, there is no satisfactory experimental animal host for unmodified virus, and infection experiments have been carried out with human volunteers.

Disease in Man. Experimental studies have shown that, following intracutaneous inoculation of as little as ten minimal infecting doses (MID) for man, local erythema and edema develop in 3 to 5 days which are associated with local multiplication of the virus. Sabin has used this reaction for immunological studies for, when virus is mixed with appropriate amounts of antiserum prior to inoculation, the reaction does not occur. With the onset of fever, the blood may contain as much as one million MID per ml.

The incubation period is usually 5 to 8 days. The prodromal symptoms include headache, backache, and malaise, followed in 6 to 12 hours by an abrupt rise in temperature. The acute phase of the disease is characterized by headache, the severe pain in the muscles and joints which has led to the name breakbone fever, abdominal pain, constipation, and anorexia. A maculopapular eruption occurs with variable frequency which ap-

pears about the third day of the disease, spreading from the trunk to the extremities and face, and persists for 3 to 4 days. The febrile period lasts for 5 or 6 days and commonly terminates by crisis. The disease is seldom if ever fatal.

Immunity. Recovery from the infection, in either man or the experimental animals which can be infected with the virus, results in a solid immunity and the appearance of complement-fixing and neutralizing antibodies in the serum. Complement-fixing antigen may be prepared from mouse brain, and protective and neutralizing antibodies are demonstrable by challenge of immunes in the first instance, and by the neutralization test using the skin reaction in man noted above, or in mice, in the second.

The two serotypes of virus cross react in the complement-fixation test with one another, as well as with some other arthropod-borne viruses, but the neutralization test is specific and serves to differentiate the serotypes sharply. Type 1 virus includes a number of strains such as one isolated in Hawaii, another isolated in New Guinea, two Indian and three Japanese strains, while type 2 includes three New Guinea strains. Two additional serotypes of the virus were isolated, together with a number of other viruses, from children with hemorrhagic fever occurring in epidemic form in the Philippines in 1956[76]; it has been suggested that these be designated types 3 and 4.

The solid immunity is to the homologous serotype and has been shown experimentally to persist for at least 18 months. Convalescents have a considerable degree of initial immunity to the heterologous serotype of virus, but it disappears relatively rapidly. Thus, within 2 months after recovery, the heterologous immunity probably often suffices to prevent naturally acquired infection. It deteriorates thereafter so that infection between 2 and 9 months after recovery with the heterologous serotype results in a modified form of the disease which is of shorter duration, without rash, and generally less severe. Active immunization with mouse-adapted strains of the virus has given encouraging results.[168, 175]

Epidemiology. The animal reservoir of dengue virus appears to be man and various species of monkeys in which viremia occurs even though the infection is symptomless; birds do not seem to be infected. The virus is transmitted by the domestic mosquito, *A. aegypti,* as noted earlier, and also by *A. albopictus* which can exist in the bush or jungle and transmit the infection among primate hosts other than man. Epidemiological observations suggest that *A. scutellaris* and *A. polynesiensis* may also be vectors under natural conditions. It may be noted that *A. vexans, A. taeniorhynchus, C. pipiens,* and anopheline mosquitoes such as *Anoph. quadrimaculatus* and *Anoph. punctipennis* are apparently unable to transmit the infection.

Man is infective for the mosquito, *i.e.*, there is viremia, for a period including the last day of the incubation period and 3 days or more after onset of the disease, and presumably monkeys are similarly infective since viremia is demonstrable experimentally for 5 to 8 days after infection. The extrinsic incubation period is 10 days to 2 weeks, and the mosquito remains infectious for life, but there is no congenital transmission of the virus. Endemic persistence of the infection depends upon climatic conditions such that mosquitoes may survive throughout the year, together with a sufficient number of newborn mammalian host susceptibles to maintain the transmission cycle.

SANDFLY FEVER[165, 167]

Sandfly fever (pappataci fever, phlebotomus fever, three-day fever) is an acute nonfatal disease of man of viral etiology which is transmitted by a species of sandfly, *Phlebotomus papatasii.*[177] Its geographical distribution is limited by the occurrence of the vector, and it is found in tropical and subtropical re-

gions during the hot dry season. These areas include various portions of the Mediterranean littoral and the Middle and Far East. The occurrence of the disease in Austrian military personnel in the Adriatic region led to the basic studies of the Austrian military commission in 1909 which substantiated the role of *P. papatasii* as a vector, which had been indicated by earlier epidemiological studies, and established the viral nature of the etiological agent.

The virus has been found to be 40 to 60 mμ in diameter and occurs as two serotypes, the Sicilian and Naples types. It does not infect the usual laboratory animals, including primates, and is not cultivable in the chick embryo or in tissue culture. It has been established in the mouse by intracerebral inoculation of the newborn mouse, producing encephalitis with lesions occurring predominantly in the hypothalamus and midbrain, after three blind passages in the case of the Sicilian type and one blind passage of the Naples type. By the tenth passage in infant mice, the Sicilian virus produced a fatal infection in the adult mouse and was fully adapted by the twenty-fifth passage. The adapted virus did not produce disease in rhesus monkeys, rabbits, guinea pigs, or hamsters but did induce an immune response. It had also lost pathogenicity for man but produced an immunity to the nonadapted virus.

The Naples strain was not regularly pathogenic for weaned mice until the thirty-fifth passage, and by the fifty-fifth passage was fully adapted, *i.e.*, virus was found in equal titer in both newborn and weaned mice. In contrast to the Sicilian type, the mouse-adapted Naples type produced a mild disease in rhesus monkeys on intracerebral inoculation, but did not infect other experimental animals. Both serological types are cultivable in human or mouse kidney cell culture after mouse passage. A cytopathic effect and plaque formation occurs in the human cell cultures after 3 to 4 tissue culture passages, but on first passage in mouse cells.[85]

Disease in Man. The incubation period of sandfly fever is usually 3 or 4 days. There may be prodromal malaise and abdominal distress. In the febrile period the viremia is of a lower level than in dengue, with perhaps 1000 MID per ml. of virus present in the blood. The symptoms, occurring in various combinations, include nausea, headache, backache, stiffness in the neck and back, pains in the joints, sore throat, and anorexia, but there is no rash as in dengue. The febrile period lasts for 2 to 4 days, and during convalescence there may be diarrhea and weakness. Relapse occurs during convalescence in some small portion of cases. The disease is rarely if ever fatal.

Immunity. Two or more attacks of the disease may occur during the same season, and this has led to the belief that an effective immunity is not produced by the infection. Controlled experiments in human volunteers, however, have shown that there is a solid immunity to the homologous virus serotype which persists for a considerable time, but that there is substantially no cross-immunity between serotypes. The development of mouse-adapted strains of both known serotypes has made possible both complement-fixation tests, using mouse brain antigen, and mouse protection tests, and the application of both of these has fully substantiated the antigenic independence of the serotypes. Active immunization of man with mouse-adapted virus has been demonstrated experimentally, and immunization against this disease, and also dengue, may assume some practical importance under certain circumstances, as in military operations.

Epidemiology. There appears to be no vertebrate host other than man, and *P. papatasii* is the only known vector of the infection. There is some evidence which has been taken to suggest that congenital transmission of the infection in the vector may occur, but this has not been established. The seasonal occurrence and short life of the vector, together with the self-limited nature of the infection in man, has been thought to

imply some other reservoir of infection, but as yet this remains purely hypothetical.

RIFT VALLEY FEVER[146]

Rift Valley fever (enzootic hepatitis) is a disease of viral etiology which was first observed in 1930 in domestic animals and man in the Rift Valley in Kenya, South Africa. Serological evidence, *i.e.*, mouse-protective antibody, indicates that the infection is widespread in Africa, occurring in Uganda, the Sudan, French Equatorial Africa, and the Union of South Africa, as well as in Kenya.

The virus is 23 to 35 mμ in size and is almost uniquely stable, serum remaining infective for as long as 2 years at refrigerator temperatures. It is cultivable in chick embryo tissue culture and in the chick embryo and is pathogenic for most experimental animals except the guinea pig, but it does not infect chickens, canaries, or pigeons. Mice are especially susceptible, and the experimental infection is fatal within 4 days. South American monkeys are somewhat more susceptible than African monkeys and respond with a febrile reaction, while the infection in African monkeys is symptomless although there is viremia. Neurotropic strains of the virus have been produced by intracerebral passage in mice. A variant, described as Lunyo virus, has been isolated from mosquitoes and a monkey in Uganda which, unlike RVF virus, produces an encephalitis in mice associated with intranuclear eosinophilic inclusion bodies.[218] This naturally occurring neurotropic strain could be made viscerotropic by intraperitoneal passage in the mouse.

Natural infection occurs in cattle and sheep, but not horses, with a 10 to 20 per cent mortality in adults and abortion in pregnant ewes. Young lambs are very susceptible to infection, and the mortality rate may be as high as 90 per cent. The characteristic lesion seen at autopsy is focal necrosis in the liver, beginning, like that of yellow fever, in the midzone of a lobule. The degeneration is of a hyaline type, and isolated cells are similar to the Councilman bodies seen in yellow fever. In the fulminating disease in lambs the destruction is very extensive and may involve practically all of the parenchymatous cells.

Naturally-occurring disease in man is found in persons associated with infected animals, such as shepherds and veterinarians. The disease seems to be especially infectious for laboratory workers, and there have been a considerable number of such infections. The incubation period is 5 to 6 days, and the onset of the disease is abrupt. It resembles dengue, with pain in the extremities and joints that may be extreme. The febrile phase lasts only a few days, convalescence is rapid, and recovery complete, and the disease is rarely fatal. In both man and lower animals a solid immunity results from the infection, and both complement-fixing and neutralizing antibodies are present in the serum.

The disease is transmitted among animals by blood-sucking arthropods which feed during the night. The mosquito *Eretmapodites chrysogaster* has been found naturally infected in the Semliki forest in Uganda and shown to transmit the disease under experimental conditions. *A. caballus* and *C. theileri* appear to be the natural vectors of the infection in South Africa.[193] Epidemiological observations suggest a wild animal reservoir of infection, but such a reservoir has not been found, and it is not known how the infection is maintained in interepidemic periods. The disease seems not to be transmitted among susceptible animals by contact, and the way in which accidental laboratory infections are acquired is unknown.

RUSSIAN SPRING-SUMMER ENCEPHALITIS[124, 180, 190, 199]

Russian spring-summer encephalitis, or RSSE (Russian Far East encephalitis,

Russian forest-spring encephalitis, Russian tick-borne encephalitis, Russian vernal encephalitis, Russian endemic encephalitis), is not to be confused with Russian autumnal encephalitis which has been shown to be Japanese B encephalitis. It was observed in 1937 in the far eastern provinces of the Soviet Union and has subsequently been found in other parts of Russia, in Central Europe, including Czechoslovakia, Bulgaria, Poland, Yugoslavia, Austria, and in Germany.

The virus is 15 to 25 mμ in diameter and may be grown on the allantoic membrane of the embryonated egg and in cultures of human embryo muscle tissue with the production of cytopathology. The mouse may be infected by subcutaneous as well as intracerebral inoculation and is extremely susceptible to mouse-adapted virus. The virus is pathogenic for sheep and rhesus monkeys, but not for guinea pigs or rabbits. A number of wild birds and rodents may be infected experimentally to give a symptomless viremia, and wild rodents have been found to be naturally infected.

Disease in Man. The incubation period is 10 to 14 days, and the febrile disease in man is characterized by signs and symptoms of a meningo-encephalitis or polioencephalitis. It varies in severity from an abortive type of perhaps a week's duration terminating in complete recovery, through a moderately severe, and more prolonged, type of encephalitis with a 20 per cent case fatality rate, and often neurological sequelae in survivors, to a severe fulminating type of meningo-encephalitis with death occurring within a week. The over-all case fatality rate is about 30 per cent, but there is a high incidence of antibody in human populations in endemic areas, and it is probable that many infections are symptomless or subclinical.

The gray matter of both brain and cord is involved, with neuronal necrosis and neuronophagia, and more marked glial and mesodermal reactions than occur in the other viral encephalitides. The disease is characterized by evidence of bulbar involvement and a peripheral type of flaccid paralysis, most often of the brachial and cervical muscles, which may be residual. In fatal cases parenchymatous degenerative changes may be found in the heart, kidney, and liver. The virus is present in the blood and spinal fluid during the course of the disease and may be isolated from the brain at autopsy.

Immunity. An immune response is evident 2 to 3 weeks after onset of the disease as complement-fixing and neutralizing antibodies in the serum. The solid immunity to reinfection is long lasting and associated with the presence of neutralizing serum antibody which reaches peak titer in 2 to 3 months. Russian workers have used formalin-inactivated brain and chick embryo vaccines for active immunization and recommend the administration of hyperimmune goat serum on exposure of susceptible persons.

Epidemiology. The first cases of this disease to be reported were seen in persons whose work took them into forested regions, but it is now clear that the distribution of the disease is less restricted, and it has occurred, sometimes in high incidence, in populated areas with greater numbers of infections in children. As noted above, the incidence of serum antibody may be relatively high in endemic areas, and human infection is doubtless more common than the number of clinical cases of the disease would indicate. Whether or not human infection may, under some circumstances, constitute a significant element of the reservoir of infection is open to question. The infection occurs in goats, and these, and possibly other, domestic animals may be a source of human infection. Human infection may occur by the alimentary route[126] and infection, especially in urban areas, from the milk of infected goats undoubtedly contributes to both human disease and to inapparent but immunizing infections.[153] It is generally believed that the significant reservoir of infection is in wild mammals and birds[81] in which viremia occurs, but the infection is otherwise essentially symptomless.

The infection is transmitted by ticks,

of which *Ixodes persulcatus* is regarded as the principal vector. The infection is congenital in these ticks, infection rates as high as 40 per cent have been found in certain small areas, and there is little doubt that there is an arthropod reservoir of infection. Natural infection of other ticks, *Haemaphysalis concinna, Dermacentor sylvarum,* and so-called gamasid ticks or mites found in bird and rodent nests, has been found also.

Human infection is acquired in forests from infected ticks which act as both a reservoir of infection and a vector in the maintenance of animal reservoirs of the virus. Infection occurring within populated areas may be transmitted in other ways. Infection by ingestion of goat's milk, as noted above, occurs and familial outbreaks of the disease have been described. It has also been found that oral transmission occurs within pheasant flocks, and this virus apparently has an appreciable potential for infection by ingestion.

Louping Ill

Louping ill is an encephalomyelitis of sheep characterized by cerebellar ataxia and necrosis of the Purkinje cells of the cerebellum similar to that observed in Japanese B encephalitis. The term louping (leaping) refers to the peculiar gait of affected animals. The disease occurs in the spring in Scotland and the northern border counties of England. Human infection has been found in Northern Ireland[127] where it has been confused with poliomyelitis, and in Sweden.[226]

The virus is 15 to 22 mμ in diameter and may be grown in chick embryo tissue culture and on the chorioallantoic membrane of the embryonated egg to produce death of the embryo. It produces fatal encephalitis on intracerebral inoculation in mice, and laboratory and field mice (voles) may be infected by peripheral inoculation. Rhesus monkeys, hamsters, and rats may be infected, the last giving a symptomless infection with viremia. Rabbits and guinea pigs are resistant to infection.

Human infections have occurred in laboratory personnel, and in both man and the sheep the disease is diphasic. In the first phase there is viremia, and in man it is accompanied by fever, headache, malaise, gastrointestinal disturbance, and prostration. After clinical improvement there is a recrudescence with fever and signs and symptoms of encephalitis. Recovery has been complete in the human cases which have been observed.

The virus is transmitted by the tick *Ixodes ricinus.* Ticks may be infected by sheep during the viremic stage of the disease, and the infection is congenital in the tick. Whether there are mammalian reservoirs of infection, such as Microtus, is not clear.

Neutralizing and complement-fixing antibodies are produced in response to infection and to immunization with vaccine. There is almost complete cross-protection between this virus and that of RSSE. The two viruses are not identical immunologically and, according to Casals,[27] differences are evident as somewhat higher homologous antibody titers and a significant difference in protection when the challenge inoculum is given peripherally rather than intracerebrally in mice. There would appear also to be differences in the pathogenicity of the two viruses for man.

Kyasanur Forest Disease[148, 224]

This disease appeared in the Kyasanur forest in the Shimoga district of Mysore, India, in 1956, and was studied for the first time in 1957. Widespread deaths occurred in native monkeys, the langur monkey (*Presbytis entellus*), and the bonnet monkey (*Macaca radiata*), and there was associated disease in man. During the summer (February-April) of 1957 the disease extended over an area of about 500 square miles, and there were 500 human cases of the disease with seventy deaths.

The disease in man does not simulate encephalitis but rather involves the hematopoietic system. Hemorrhage is the obvious major clinical symptom, with evidence of massive hemorrhage in

the chest cavity in fatal cases. The source of infection appears to be monkeys,[95] and possibly rodents are infected also. The infection is not transmitted by mosquitoes, but there is reason to believe that Haemaphysalis ticks may be the vector.[213] An accidental infection occurred in the laboratory in the absence of an insect vector.

A virus was isolated from monkeys and also from the blood in human cases of the disease by Work, Trapido, and their colleagues.[223, 225] The viremic period in man is relatively prolonged, and the blood has been found to be infectious from 2 days before to as long as 10 days after onset of the disease. The KFD virus is cultivable in chick embryo tissue culture and produces cytopathogenic effects, and the mouse may be infected by intracerebral inoculation to produce a disease in 3 or 4 days characterized by prostration, hind limb paralysis, and tremors. Antibody-fixing complement in the presence of mouse brain antigen and neutralizing antibody demonstrable in the mouse are found in convalescents. The virus appears to be antigenically closely related to, but not identical with, the RSSE virus.

A similar hemorrhagic disease, Omsk hemorrhagic fever, has been described in Russia[125, 130] as of similar etiology. Observations such as these suggest that the virus of RSSE is a member of a group or complex of closely related viruses, some of which produce encephalitis in man with the characteristic paralysis described above, but louping ill virus infections in man are not predominantly encephalitic, and infections with still other of these viruses are characterized by hemorrhagic symptoms.

Powassan Virus

Powassan virus was isolated from a fatal case of encephalitis in Ontario in 1958 by intracerebral inoculation of suckling mice.[137] It proved to belong to hemagglutinin group B (see below) and closely related to RSSE,[29] the first member of the group to be isolated in the Americas. It was not found in ticks or mosquitoes, but there is serological evidence of infection in chipmunks and squirrels.[136, 138] Apparently the same virus was isolated from pools of *Dermacentor andersoni* collected in Colorado some years earlier, but not identified until the Powassan isolate was described.[208]

COLORADO TICK FEVER

Colorado tick fever (American mountain fever, mountain fever, mountain tick fever, tick fever, nonexanthematous tick fever) occurs in the Rocky Mountain regions of the United States where the tick vector is prevalent.[24, 25, 46] The disease in man was described as a clinical entity in 1930 but probably accounted for some part of febrile disease reported by military personnel in that area in earlier years.

The viral nature of the etiological agent was established by serial transmission of the disease in man and in hamsters by the parenteral inoculation of blood, serum, and filtered preparations in 1944. In 1947 it was adapted to the mouse by intracerebral passage in the suckling animal, and the mouse-adapted virus grown in the yolk sac of the embryonated egg where the virus is found largely in the central nervous system of the embryo. Such embryo-adapted strains are of reduced virulence for man. It has also been grown in cultures of KB epithelial carcinoma cells with the appearance of scattered foci of pathological changes in the cell sheet which spread progressively.[152] The virus has been isolated directly from infected human or monkey blood or from ticks by this tissue culture method. There is some discrepancy in the reported size of the virus; according to one report it is extremely small, 10 mμ, and to another 35 to 50 mμ. It is more stable on storage than many viruses.

On intracerebral inoculation of mice and hamsters, the viruses produces lesions similar to those of louping ill virus,

i.e., subcortical vascular engorgement and petechial hemorrhages in the cerebrum and destruction of the Purkinje cells.[145] Cytoplasmic inclusion bodies are found in the Purkinje cells and in the neurones of the hippocampus and pons. The experimental infection in the rhesus monkey, by the intravenous or subcutaneous routes, is essentially asymptomatic but viremia occurs and persists for 4 to 5 weeks.[61]

Disease in Man. The disease in man is diphasic and is characterized by a sudden onset with chills and aching, and the symptoms include headache, backache, anorexia, nausea, and muscular pains. With the appearance of symptoms there is a febrile reaction which persists for about 2 days, a period of remission occurs, with recrudescence in 2 or 3 days. Convalescence may be relatively prolonged but is without complications, and recovery is complete; no deaths from the disease have been reported.

Immunity. Complement-fixing[205] and neutralizing[60] antibodies are produced in response to infection, appearing by 2 weeks after onset of the disease, and persisting for at least as long as 3 years. Experimental inoculation of human volunteers has shown that here is a solid immunity to reinfection. Active chick embryo–adapted virus used as an immunizing agent produces a solid immunity, but mild symptoms of the disease occur in some persons.

Epidemiology. The wood tick, *Dermacentor andersoni,* is naturally infected with the virus, the infection is congenital, and the tick is a reservoir of infection.[162] The virus has been isolated from the dog tick, *D. variabilis,* on Long Island, but no human cases of the disease have been recognized there, and *D. andersoni* is considered to be the primary, and possibly sole, vector of the infection. The virus also occurs in a vertebrate reservoir of infection, in the Colombian ground squirrel, *Citellus comunbianus,* as indicated by serological evidence, and has been isolated directly from the golden-mantled ground squirrel, *Citellus lateralis lateralis,* and from the porcupine, *Erethizon dorsatum epixanthum.*[22, 23, 47] Human infection is acquired from infected ticks, and tends to occur in persons exposed to tick bites by occupation, as in adult males.

EPIDEMIC HEMORRHAGIC FEVER[132, 181, 196, 222]

There are a number of hemorrhagic fevers in Asia, of which epidemic hemorrhagic fever is one. It was recognized by the Japanese in Manchuria in 1939 and appeared among United Nations military personnel in Korea in 1951 when approximately 1000 cases of the disease occurred. It was shown by both Russian and Japanese workers that the disease could be transmitted to human volunteers by the parenteral inoculation of blood or urine taken early in the febrile period of the disease, that the causative agent passed bacteria-proof filters, and that convalescent serum is protective. While this evidence is taken to indicate that the disease is of viral etiology, it has not been possible to produce infection in experimental animals or to culture a virus by the usual methods, and the etiology remains somewhat uncertain.

Infection in man results in an acute febrile disease with abrupt onset and headache, chills, anorexia, and vomiting. A petechial rash develops, usually on the palate and axillary skin folds, and there is often severe albuminuria. There may be thrombocytopenia of sufficient severity that there is associated purpura and hematuria. The capillary epithelium is affected, resulting in capillary stasis, transudation, and a tendency to hemorrhage. The microscopic changes are most prominent in the kidney, but focal necrosis and mononuclear infiltration may be found in other organs. The fatality rate is variable, ranging from 5 to 14 per cent.

The disease tends to occur in two peaks during the year, the one in late spring and the other in the autumn, and the infection appears to be contracted within relatively sharply defined foci in rural areas, often along river banks and

on swampy ground. The epidemiological character of the disease is consistent with that of an arthropod-borne infection existing as an animal, possibly rodent, reservoir of infection, a conclusion reached by Japanese, Russian, and American workers.[58] The delimited areas in which infection occurs are regarded as suggestive of a mite vector. In the absence of definite information, however, the epidemiology of the disease remains conjectural.

An overtly similar disease observed in the Philippines and Thailand in young children and occurring in epidemic form is known as Philippine hemorrhagic fever and Thai hemorrhagic fever.[156] Three virus strains, KLA 16, TH 35 and BaH 306, were isolated in hamster kidney and rhesus monkey kidney cell cultures[76] which proved to be related antigenically to the Chikungunya virus and members of group A (see below). One, KLA 16, produces hemorrhagic disease in rodents.[72] Rise in antibody titer in paired serums suggests an etiologic relation to the disease, but differences in the ability to produce hemorrhagic disease under experimental conditions are not yet resolved.

During an outbreak of febrile hemorrhagic disease in Argentina in 1958 a virus, designated Junin virus, was isolated. It may be carried by intracerebral passage in mice, and grows in HeLa cell culture producing a characteristic cytopathic effect, and basophilic bodies occur in the cytoplasm of the cells.[142] It is a member of the arbor virus group and is ungrouped serologically.

OTHER ARTHROPOD-BORNE ANIMAL VIRUSES[41, 101, 183]

A number of other viruses with neurotropic potentialities have been isolated by intracerebral inoculation of the infant mouse, from mosquitoes, and in a few instances from man associated with febrile disease, in this country, in South America, and in Africa. Some of these have been named according to the mosquitoes from which they have been isolated, such as Anopheles A and Wyeomyia; others by the locality in which they were found, such is Ilheus, Ntaya, and Sindbis; still others have been given code names, and others, Chikungya and O'nyong-nyong, are given the descriptive native name of the disease in man. In a few instances they have been found to be identical, or nearly so, with previously known viruses, but for the most part they are distinct entities. Some of these viruses are described below.

NORTH AMERICAN ISOLATES

Two viruses have been isolated from mosquitoes in the western part of the United States, the California, or Hammon-Reeves, virus, and the Turlock virus.

California Virus.[74, 75, 158] Three strains of this virus were isolated in 1943 from pools of *A. dorsalis* and *C. tarsalis* by intracerebral inoculation of young mice; they produced encephalitis in these animals, cotton rats, and hamsters. Inapparent infection with viremia was produced in ground squirrels, but calves and chickens were not susceptible. The virus grew well in the chick embryo and in other parts of the embryonated egg, but usually without death of the embryo. It was found to be 60 to 125 mμ in diameter and immunologically distinct from the other arthropod-borne viruses.

This virus was found only in the San Joaquin Valley in California, and within Kern County there was serological evidence of infection in man, horses, cattle, rabbits, and ground squirrels. This virus was the probable cause of fatal encephalitis in an infant, and the possible cause of disease in two other persons, but has not been isolated from man or other mammals. It is believed that it occurs as

a reservoir of infection in lower animals, and the infection is maintained, and transmitted to man, by mosquitoes to give a symptomless or subclinical infection.

Turlock Virus.[121, 122, 219] This virus was isolated from *C. tarsalis* in California by intracerebral inoculation of infant mice. It grows in the embryonated egg, killing the embryo in 2 to 6 days and may be cultivated in chick embryo cell culture. It was found to be relatively large, 120 to 180 mμ in diameter, and, by the complement-fixation reaction, unrelated to other arthropod-borne viruses. It is not known to cause disease in man.

Cache Valley Virus. This virus was isolated from a pool of *Culiseta inornanta* collected in the Cache Valley in northern Utah by intracerebral inoculation of weanling mice,[88] and can be grown in hamster kidney cell culture to produce cytopathic effects. There is serologic evidence of infection of horses in the area, but not in man. The virus has also been found in Brazil, and was isolated from a pool of *Aedes scapularis* Trinidad.[44] In Trinidad there was serologic evidence of infection in horses and monkeys (Alouatta), and in one third or more of human serums examined. The virus is antigenically related to the Bunyamwera group and has been placed in it.

SOUTH AMERICAN ISOLATES

Of the group of viruses isolated in South America and producing encephalitis in suckling mice, all but one, Mayaro virus, have been isolated from mosquitoes and have not been found in mammalian hosts.

Anopheles Viruses. The viruses designated Anopheles A and Anopheles B were isolated in Colombia in 1940 by Roca-García[160] from *Anopheles boliviensis*. They are of the same size, 70 to 120 mμ in diameter, but are antigenically distinct entities and differ in their pathogenicity for the embryonated egg. Roca-García was able to grow the Anopheles A, but not the Anopheles B, virus in the embryonated egg. Subsequently Taylor was able to grow the mouse-adapted Anopheles B virus in the embryonated egg after serial passage by intracerebral inoculation of the embryo. Neither virus grows well on the chorioallantoic membrane, but both are found in the brain of the embryo after inoculation into the yolk sac or amnionic sac; the A virus is found in the brain following allantoic cavity inoculation, but the B virus would not grow when inoculated by this route.

Wyeomyia Virus. This virus was also isolated by Roca-García in Colombia in 1940, but from *Wyeomyia melanocephala*. It is similar in size to the Anopheles viruses, 70 to 120 mμ in diameter, and produces encephalitis in the infant mouse following intracerebral inoculation. It differs in that it is feebly pathogenic for the chick embryo, growing only when inoculated intracerebrally, and will not grow in other parts of the embryonated egg. It is immunologically distinct from the Anopheles and other arthropod-borne viruses.

Ilheus Virus. The Ilheus virus was isolated in 1944 in Brazil by Laemmert and Hughes[118] from pooled Aedes and Psorophora mosquitoes. It is also found in Panama where birds may be hosts.[57] It is cultivable on the chorioallantoic membrane of the embryonated egg but disappears from this site relatively rapidly. The embryo is infected following yolk sac inoculation, and the virus is found in highest concentration in the brain. It is highly pathogenic for mice, even when inoculated by other than the intracerebral route. It is smaller than the other South American mosquito isolates, 15 to 30 mμ in diameter. It is a distinct immunological entity but is of particular interest in this respect because it is antigenically related to the SLE, JBE, and West Nile viruses.

Encephalomyelitis Viruses. The Haemagogus A, Haemagogus B, Sabethes, and Leucocelaenus viruses were isolated in 1945 and 1946 from the indicated mosquitoes in the Passos area of Brazil by Hughes and Perlowagora.[93] These viruses have been found to be

extremely closely related to one another, and they have been shown by Smithburn[38] and Kerr[38] to be identical with the GD7 and FA strains of mouse encephalomyelitis virus (Chap. 39) by neutralization and complement-fixation tests respectively.

Mayaro Virus. A febrile disease of viral etiology occurred as an outbreak in Mayaro county, Trinidad, and Casals and Whitman[31] reported the isolation of the causative virus by intracerebral inoculation of sucking mice in 1957. The Mayaro virus is closely related to the Semliki forest virus (see below) found earlier in Africa and, if not identical with it, is a closely related variant. An outbreak of febrile disease of apparently the same etiology occurred in the region of the Guama River in Brazil, and surveys have shown that antibody to this virus, and the Semliki virus, is widely distributed in the Amazon region.

Other Isolates. New isolates continue to be described, and those from South America include the following:

Bussuquara virus was isolated from a sentinel howler monkey (*Alouatta beelzebul*) in Belem, Brazil, by intracerebral inoculation of suckling mice.[66] It is a member of group B but is only distantly related to other members of the group.

Guaroa virus was isolated in the Orinoco river basin in Colombia from the blood of persons having febrile disease.[68] It has a one-sided antigenic relationship to California encephalitis virus by hemagglutination-inhibition, but is unrelated by the complement-fixation reaction.

Uruma virus was isolated from the blood of immigrant Okinawans settled in a rain forest in Colombia and ill of epidemic febrile disease.[176] The virus is small with an estimated size of 17 to 26 mμ and cultivable in the yolk sac of the embryonated hen's egg and in hamster kidney, KB, human liver and monkey kidney cell culture with cytopathic effects, but not in HeLa cell culture. It is antigenically related to Mayaro, Semliki forest, equine encephalomyelitis and other viruses making up group A.

Manzilla virus was isolated from howler monkeys (*Alouatta seniculus insularis*) in Trinidad.[2] It is cultivable in the yolk sac of the embryonated hen's egg. It is antigenically unrelated to the other known arbor viruses and is included with the ungrouped viruses.

Kairi virus was isolated as 11 strains from pools of various kinds of mosquitoes in Trinidad[1] by intracerebral inoculation of suckling mice. The virus could be adapted to produce fatal infection in adult mice and most strains could be adapted to the chick embryo and hamster kidney cell culture. The virus is antigenically related to Bunyamwera virus and is included in that group.

Oropouche virus was isolated from the blood of a man with febrile illness in Trinidad.[3] Suckling mice could be infected by the intraperitoneal as well as intracerebral route, the hamster by both routes, and the guinea pig by the intracerebral route. Mouse passage virus could be propagated in the embryonated hen's egg by yolk sac or amnionic inoculation. The virus is antigenically related, by neutralization and complement-fixation tests, only to the Simbu virus of African origin.[217]

AFRICAN ISOLATES

As in South America, a number of viruses neurotropic for infant mice on intracerebral inoculation have been isolated from mosquitoes without apparent relation to human disease, and also from human cases of febrile disease in Africa. One of the first of these viruses to be found was the Bwamba fever virus, which was isolated in 1937 from persons suffering from febrile disease, and this was followed by a series of mosquito isolates, and the isolation of the virus of the dengue-like disease Chikungunya and others from humans with febrile illness.

Bwamba Fever Virus. Bwamba fever is a mild febrile disease of man which occurs in African natives in Uganda. The disease is characterized by abrupt onset with fever, headache, backache,

mild conjunctival injection, and rash. The disease persists for only a few days, and convalescence is rapid. Nine strains of the causative virus were isolated from the blood[189] by intracerebral inoculation of mice, producing hyperemia and edema of the brain with degenerative changes in the pyramidal cells of the cortex. The virus is almost completely avirulent for the mouse by peripheral inoculation. A nonfebrile infection is produced in rhesus monkeys, and guinea pigs and rabbits are not susceptible to infection. The virus is cultivable in the embryonated egg by yolk sac inoculation and is found in highest concentration in the brain and body of the embryo. The embryo is not killed, but evidence of gross hemorrhage is found in the brain. The virus is 70 to 120 mμ in diameter and is antigenically distinct. It is antigenically related to Pongola virus.[115] Neutralizing antibody occurs in the native population in proportions ranging from 13 to 81 per cent of serums examined, suggesting that the infection is widespread.

Semliki Forest Virus. This virus was isolated in 1942 from *A. abnormalis* in the Semliki forest of western Uganda by Smithburn and Haddow.[185] It is pathogenic for the mouse by peripheral as well as by intracerebral routes of inoculation and produces fatal infections in guinea pigs, rabbits, and rhesus monkeys on intracerebral inoculation. The brain lesions in infected animals are similar to those produced by the equine encephalomyelitis viruses. The embryonated egg may be infected by various routes of inoculation, including the chorioallantoic membrane, and the infection is uniformly fatal to the embryo. The titer of virus in the body of the embryo is as high as or higher than in the brain, generalized congestion and localized hemorrhages are found in both the brain and body, and the virus appears to be more pantropic than neurotropic in the embryo.

The virus is 30 to 40 mμ in diameter.[36, 37] It is antigenically distinct and, as noted above, the Mayaro virus found in Trinidad is closely related to it. The Kumba virus, isolated from Eratmapodites mosquitoes in the Kumba region of the British Cameroons in West Africa has been found to be antigenically identical with it. While the Semliki and Kumba viruses have been found only in mosquitoes, the pathogenicity of the virus for man is shown by the occurrence of human disease in Trinidad and possibly elsewhere in South America, and it is apparent that the infection is widespread in the tropical regions of both continents.

Ntaya Virus. This virus was isolated[186] in 1943 from pooled mosquitoes of several genera and species, including Culex and Aedes, collected in the Ntaya swamp in western Uganda. The infection was uniformly fatal to mice by intracerebral inoculation after the eleventh passage, but the intranasal and intraperitoneal routes of inoculation were not effective. Infections uniformly fatal to the embryo are produced by inoculation of the yolk or amnionic sacs, but embryos die only irregularly following inoculation on the chorioallantoic membrane or allantoic cavity. The virus is neurotropic in the embryo, with higher concentration of the virus in the brain than in other parts of the body, and the gross hemorrhagic lesions are confined to the brain. The virus is 70 to 120 mμ in diameter and antigenically distinct but has been found to give some cross-reaction with Ilheus virus in the complement-fixation reaction. There is no evidence of naturally-occurring mammalian infection, and persons living in the vicinity of the swamp did not show serum antibody.

Bunyamwera Virus. This virus was isolated in 1943, from Aedes mosquitoes caught in an uninhabited part of the Semliki forest, by the intracerebral inoculation of mice.[187] It produces a lethal infection in hamsters inoculated by the intracerebral or subcutaneous routes but not in guinea pigs by the intraperitoneal route. Embryonated eggs may be infected by yolk sac or amnionic cavity inoculation, and the embryo is killed. Gross lesions are confined to occasional hemorrhage in the brain where

the virus is present in higher concentrations than in the body, and the virus is neurotropic in the embryo. It is of the same order of size as the Ntaya virus, 70 to 120 mμ, and is antigenically distinct. While naturally-occurring infections have not been recognized, the virus is pathogenic for man as shown by the occurrence of disease, one an encephalitis, in two of four volunteers inoculated,[191] and naturally-occurring infection has been observed.[112]

Uganda S Virus. The Uganda S virus was isolated in 1947, in Bwamba county, Uganda, from a pool of mosquitoes containing *A. longipalis, A. ingrami,* and *A. natronius,* by intracerebral inoculation in the mouse.[42] Hamsters may be infected by the intracerebral route, but the infection is not uniformly fatal. The virus may be grown in the embryonated egg by yolk sac and amnionic sac inoculation, reaching higher concentrations in the brain than in the body of the embryo. The embryos are usually not killed, and the gross lesions are minor, consisting of congestion and some small hemorrhages in the brain. The virus is 15 to 30 mμ in diameter, and antigenically distinct, but gives irregular cross-reactions in the complement-fixation test with some yellow fever virus antigens. Neutralizing antibody has been found in human serums, with 7.7 per cent positives found in Uganda and Tanganyika, and 35 to 50 per cent in Nigeria. On this basis, the virus would seem to be one of the most common in Nigeria.

Zika Virus. This virus was isolated in 1947 and 1948, first from the blood of a captive monkey (yellow fever sentinel) in the canopy of the forest near Zika, Entebbe, Uganda, and again from a pool of *A. africanus* in the same region.[43] It was isolated in mice by intracerebral inoculation but is not pathogenic for hamsters or for mice by the intraperitoneal route. It is cultivable in the embryonated egg by inoculation into the yolk, amnionic, and allantoic sacs but usually does not kill the embryo; gross lesions are uncommon, and the virus is pantropic rather than neurotropic in the embryo. The virus is 15 to 30 mμ in diameter and antigenically distinct.

Sindbis Virus. This virus was isolated in 1955 from *C. univittatus,* and once from a hooded crow, in the Sindbis district in northern Egypt[200] by the intracerebral inoculation of infant mice. It has also been found in India.[179] A number of animals may be infected experimentally, including chickens, horses, doves, and crows, to produce viremia without symptoms, but infant mice show paralysis and die in 2 or 3 days. The virus may be grown in the embryonated egg by inoculation of the yolk sac, and the embryos die in 1 to 3 days. It may also be grown in chicken embryonic tissue culture with marked cytopathogenic effects apparent in the fibroblast outgrowth, but no cytopathogenic effects are produced in HeLa cell or human embryonic skin-muscle tissue culture.[55] The virus is 40 to 48 mμ in diameter and thus intermediate in size in relation to the viruses described above. It is antigenically distinct.

Chikungunya Virus. Chikungunya is a dengue-like disease which occurred as an outbreak of infection in the Newala district of Tanganyika in 1953. The disease in man appears to be a clinical variant of dengue in which the headache is lacking, but the eyeballs are tender and there are severe joint pains. In a study of the etiology of the disease, Ross[161] isolated two viruses, the one Chikungunya virus which is the etiological agent of the disease, and the other Makonde virus, named after the region (Makonde plateau) in which it was found, whose presence was fortuitous. The two viruses are immunologically unrelated. They also differ in their pathogenicity for the mouse in that the median incubation period of Chikungunya virus infection following intracerebral inoculation was 4 days, and 9 days in the case of Makonde virus, and the former killed in 4 to 7 days while the latter required 14 days or longer.

The Chikungunya virus was isolated from the blood of patients with febrile disease and also from *A. aegypti,* and

neutralizing antibody was found in the serum of convalescents. Rabbits and guinea pigs were apparently not susceptible to infection. The virus gave a slight cross-reaction in the neutralization test with dengue type 1, but not with type 2, and gave almost complete cross-reactions with Semliki forest virus.

Other Isolates. Other virus strains, some related to existing groups and others serologically independent, continue to be reported from Africa. Some of these isolates, not referred to elsewhere, are:

Wesselsbron virus is apparently widespread in South Africa. It is repeatedly isolated from various mosquito species and from naturally occurring infections in sheep and man,[87, 188] and serological evidence indicates that it infects man and animals fairly commonly. It is a member of group B.

Spondweni virus has been isolated from a variety of mosquitoes in Togoland. Laboratory infections, but not naturally occurring disease, have occurred in man.[113, 134] It is a member of group B.

Middelburg virus was isolated from mosquitoes during an epizootic of disease in sheep in which it appeared to be a causative agent.[110] It is a member of group A.

Germiston virus, a member of the Bunyamwera group, has a one-sided relationship by the hemagglutination reaction, but it is unrelated by the complement-fixation test. It has been isolated from mosquitoes and accidental infection has occurred in laboratory workers.[114, 149]

Witwatersrand virus was isolated from culicine mosquitoes in South Africa. Serologic evidence indicates that human infection is uncommon, and the virus is apparently unrelated to the established groups.[133]

O'nyong-nyong virus was isolated from the blood of patients during a major epidemic of febrile dengue-like disease in Uganda and Kenya, and was also found in anopheline mosquitoes. Infected suckling mice show a patchy alopecia and retardation of growth, and mouse passage virus may be propagated in chick embryo fibroblast culture, forming plaques in monolayers. In the plaque-inhibition test the virus shows a one-sided relation to Chikungunya and Semliki forest viruses, and is considered to be a member of group A.[220]

Ndumu virus was isolated from mosquitoes in Natal. Human disease has not been observed but neutralizing antibody has been found in a portion of human serums examined. The virus is unrelated to representatives of groups A and B by complement-fixation, and possibly also by hemagglutination.[111]

INTER-RELATIONSHIPS AMONG ARTHROPOD-BORNE VIRUSES[155]

The arthropod-borne viruses have in common a reservoir of infection in lower animals, a transmission cycle in which the infection is carried by biting arthropods, mosquitoes, or ticks, and a general kind of pathogenesis in which there is viremia in the bird or mammalian host and infection of the viscera or central nervous system, depending upon the relative neurotropism of the virus. As noted earlier, human infection is usually incidental to the natural history of the virus although under certain circumstances, as in Aedes-transmitted yellow fever, man may function as a significant host or even a reservoir of infection. There are also other indications that these viruses are more closely related to one another than to other viruses; for example, all the arthropod-borne viruses which have been tested are inactivated by desoxycholate in 1:1000 dilution, while other neurotropic viruses, including poliovirus,

mouse encephalitis viruses, and Coxsackie and encephalomyocarditis viruses (see below) are not.[202]

Among these viruses there are groups and subgroups of viruses which resemble one another. On the whole, they fall into two general size ranges, those 15 to 30 mμ in diameter and the larger viruses 20 to 120 mμ in diameter, although there are exceptions such as the Sindbis virus which is intermediate in size. The significance of grouping by size is uncertain, for antigenic relationships, for instance, may occur between the larger and smaller viruses.

Immunological relationships are often of basic significance and are also of practical importance in relation to cross-immunity to infection and to serodiagnosis of disease or serological identification of viruses. These viruses, like many other microorganisms, induce the formation of complement-fixing and neutralizing, or protective, antibodies which may be identical but often are not. In addition, many, but not all, of the arthropod-borne viruses agglutinate goose red blood cells, and, as in the case of influenza and related viruses, the hemagglutinin is antigenic and stimulates the formation of hemagglutinin-inhibiting antibody. The hemagglutinins and complement-fixing antigens are separable by chromatography and preliminary studies have indicated that virus strains of groups A and B have two hemagglutinins which are antigenically similar, and two complement-fixing antigens are present in strains of group B, but only one, the virus particle, in group A.[67]

Of the three general kinds of serological reactions which are available for the characterization of these viruses, the hemagglutinin-inhibiting antibody is the least specific in that it gives a high proportion of cross-reactions, neutralizing antibody is highly specific,[155] and complement-fixing antibody is intermediate in specificity.

The application of the hemagglutination-inhibition reaction allows the separation of the arthropod-borne viruses into broad groups.[38] Groups A and B, the latter being considerably the larger, were distinguished first. With the isolation of related strains, the previously ungrouped Bunyamwera virus provided the basis for a Bunyamwera group.[32] These groupings are shown in the accompanying table with virus strains illustrating the observed separations. A group C was described,[33] which consists of virus strains isolated in Belem (Brazil). Of the remainder, some fall into minor groups made up of two or three related strains, while others are as yet ungrouped. These groupings are given in detail by Casals.[30] It is probable that this grouping can be simplified somewhat by aggregation of closely related viruses as variants of a designated prototype strain.

Subgroups within the hemagglutinin groups may be distinguished by cross-reactions in the complement-fixation reaction in which the titer of heterologous reactions is commonly less than that of the homologous reaction. For example, West Nile, St. Louis encephalitis, Japanese B encephalitis, and Murray Valley encephalitis viruses are related with respect to complement-fixing antigens, and yellow fever virus is related to this group and also to dengue and Uganda S vi-

Hemagglutinin Groups of Arthropod-Borne Viruses

GROUP A	GROUP B		BUNYAMWERA GROUP
EE viruses	St. Louis E	West Nile	Bunyamwera
Mayaro	yellow fever	Uganda S	Cache Valley
Semliki	louping ill	Ilheus	Germiston
Uruma	Russian SSE	Powassan	Guaroa
Chikungunya	Kyasanur forest	Bussuquara	Ilesha
Sindbis	Zika	dengue viruses	Kairi
O'nyong nyong	Japanese B	Wesselsbron	Wyeomyia
Middelburg	Murray Valley	Ntaya	Chittoor

ruses. A Japanese B encephalitis antiserum, having a homologous titer of 1:128, may show a titer practically as high against West Nile antigen and one of 1:32 against yellow fever antigen. Or, yellow fever antiserum having a homologous titer of 1:28 may fix complement in titers as high as 1:32 in the presence of West Nile or Japanese B encephalitis antigen. Similarly, a dengue antiserum having a homologous titer of 1:128 may fix complement with yellow fever antigen in dilutions of 1:8 to 1:32, in the presence of West Nile antigen in dilutions of 1:16 to 1:64, and in the presence of Japanese B encephalitis antigen in dilutions of 1:16 to 1:64. Similar cross-reactions occur between the western and eastern varieties of equine encephalomyelitis virus.

When the more highly specific neutralizing antibodies give heterologous reactions, it is generally taken to be indicative of an extremely close relationship between the viruses. There is, for instance, a partial cross-neutralization between the western and eastern equine encephalomyelitis viruses, and a similar relationship among the West Nile, Japanese B encephalitis, St. Louis encephalitis, and Murray Valley encephalitis viruses. In the case of Russian spring-summer encephalitis, louping ill, and Kyasanur forest viruses, the relationship is very close and the differences among these viruses are considered to be of little more than variant status. Semliki forest virus, Kumba virus, and Mayaro virus give essentially complete cross-neutralization reactions and are therefore regarded as practically identical, and Chikungunya virus is either identical with or closely related to them. These relationships, together with those evident in the complement-fixation reaction, are indicated as subgroups within the hemagglutinin groups in the accompanying table. Such subgroups may not, however, fall entirely within the hemagglutinin groups; the Chikungunya virus, for instance, although closely related to Semliki forest virus, also cross reacts to a minor degree with dengue type 1 virus.

OTHER NEUROTROPIC VIRUSES

There are still other neurotropic viruses which exist as reservoirs of infection in lower animals and infect man to produce disease, but which do not depend upon an insect vector for transmission of the infection. These include lymphocytic choriomeningitis, the encephalomyocarditis group of viruses, and rabies.

LYMPHOCYTIC CHORIOMENINGITIS

This disease was first observed in 1934, by Armstrong and Lillie,[8] who isolated the virus in a monkey being used in the study of St. Louis encephalitis. The choroid plexuses and meninges were involved, and there was lymphocytic infiltration, these characteristics giving the disease its name. The following year it was found by Traub[212] to be widespread as an endemic, often symptomless, infection in mice, and in 1936 the virus was isolated, both in this country and in England, from human cases of disease diagnosed as aseptic meningitis.

The virus is 40 to 60 mμ in diameter and occurs as a single antigenic type. It may be grown in mouse or chick embryo tissue culture[16] and in the embryonated egg on the chorioallantoic membrane or in the yolk sac. In the egg it does not kill the embryo, and the egg may hatch and the chick survive.

Disease in Man.[123, 182] The human disease occurs during the cold months of the year, chiefly in adults 20 to 40 years old. It varies in severity from a

mild influenza-like disease, through aseptic meningitis or meningo-encephalitis, to a fatal systemic form of the disease. The respiratory type of disease may not progress further, and few such cases are recognized as infections with this virus. Or the respiratory symptoms may be prodromal, and the disease progress with the appearance of central nervous system involvement within a few days, including sudden severe headache, fever, nausea, and drowsiness, or the meningitis may occur without prodromata. In the severe form of the disease, which occurs relatively rarely, the symptoms are referable to the portions of the central nervous system affected. During the febrile stage of the disease the virus is present in the blood, nasopharyngeal secretions, and urine, and in the spinal fluid in the meningeal type of disease. Complications are rare and recovery is usually complete.

Immunity. Both complement-fixing and neutralizing antibodies are formed in response to the infection. Both appear relatively late in the disease, complement-fixing antibody reaching diagnostic levels in 3 to 4 weeks and disappearing within a few months, and neutralizing antibody reaching diagnostic titer in 7 to 8 weeks after onset of the disease and persisting for a period of years.

Experimental Infections. A number of experimental animals are susceptible to infection, including mice, guinea pigs, dogs, monkeys, and chimpanzees, but rabbits and birds are resistant. The mouse and guinea pig are the experimental animals of choice. The mouse may be infected by intranasal or intracerebral inoculation to produce a fatal infection; tremors and convulsions develop in 1 to 2 weeks with death 2 or 3 days later. Peripheral inoculation results in a nonfatal infection unless very large doses of virus are given by the intraperitoneal route. Fatal disease is produced in the guinea pig by intracerebral inoculation and may also occur following peripheral inoculation. In both kinds of animals virus is present in the nasal secretions, urine, and feces, and postmortem findings include areas of lymphocytic infiltration in the central nervous system and in the organs, and there may be hepatitis.

Epidemiology. Mice, dogs, and monkeys have been found to be naturally infected with the virus, and it is probable that human infection is most often contracted from mice. The mouse infection is endemic, with infection transmitted *in utero* or shortly after birth and maintained by healthy carriers.[212]

It is not clearly established in what way man contracts the infection from the mouse or other animal. Arthropods, including culicine mosquitoes, stable flies, wood ticks, and body lice, are capable of transmitting the infection under experimental conditions, and it has been shown also that the virus may be transmitted by infected larvae of *Trichinella spiralis,* but the relative importance of such transmission under natural conditions is not known. It is generally believed that the portal of entry is usually the respiratory tract and that the infection is acquired by inhalation; laboratory infections have occurred which were probably acquired in this way.

The infection in man is doubtless much more common than the number of clinical cases of the disease suggest. Armstrong found that 11 per cent of 2000 persons examined at random had neutralizing serum antibody, and it is probable that many immunizing infections are symptomless or are assumed to be influenza or related respiratory disease.

Durand's Disease

This is a febrile disease characterized by headache, upper respiratory, meningeal, and gastrointestinal symptoms which Durand[45] described as occurring in himself and in two human patients who were inoculated for pyrotherapeutic purposes. Later Findlay[53] acquired the disease as a consequence of a laboratory infection.

During the febrile stage of the disease, lasting for less than a week, the blood is infective for the guinea pig by intracere-

bral or other routes of inoculation. The experimental disease is characterized by adenopathy, pneumonitis, and enlargement of the spleen, and the fatality rate is about 10 per cent. Mice, hamsters, dogs, cats, and monkeys are also susceptible to experimental infection. In both man and experimental animals complement-fixing and neutralizing antibodies appear in the serum after about a week. The virus is 40 to 60 mμ in diameter, cultivable in chick embryo tissue culture, and on the chorioallantoic membrane of the embryonated egg, to give an infection which is lethal to the embryo. It is immunologically unrelated to the virus of lymphocytic choriomeningitis.

ENCEPHALOMYOCARDITIS VIRUSES

The encephalomyocarditis, or EMC, viruses are represented by four strains which appear to be substantially identical in immunological and other respects. The first to be isolated, the Columbia SK virus, was found in 1940 in a monkey which had been inoculated with the Yale SK strain of poliovirus, but it is clearly distinct, both in antigenicity and pathogenicity, from poliovirus. The MM virus was isolated in 1943 from the brain of a hamster which had been inoculated with material from a human case of paralytic disease. These two strains were at one time considered to be "mouse poliomyelitis viruses" or murine variants of poliovirus, but it is now clear that this is not true. The strain called the EMC virus was isolated in 1945 from a captive chimpanzee in Florida which had died of acute endocarditis complicated by myelitis. The Mengo virus was isolated in 1948 from a captive monkey in Uganda and later from mosquitoes (*Taeniorrhynchus fuscopennatus*) and a mongoose in the same area. It has also been found as the causative agent of an epizootic disease of swine in Panama.[147]

These viruses are about 25 mμ in diameter, values above and below this being given by different methods of measurement. A variety of experimental animals may be infected to produce disease ranging from severe fatal disease to inapparent infection, depending upon the species and age of the animal.[102] Rapidly fatal disease is produced in the mouse, hamster, and cotton rat, disease varying in severity in the guinea pig and rhesus monkey, and inapparent infection with no more than a transient febrile reaction in the rabbit and white rat. In experimental animals the virus is pantropic, occurring in the blood and widely distributed in the tissues, including the central nervous system. Degenerative and inflammatory changes are produced in the skeletal and cardiac muscle, and the lesions in the central nervous system are those of polioencephalitis, with congestion, lymphocytic infiltration, and focal areas of necrosis in the granular and Purkinje cell layers. The virus is cultivable in the chick embryo, killing the embryo in 3 or 4 days. In mouse muscle tissue culture a characteristic cytopathic effect is produced, with essentially complete destruction in 72 hours.[35]

Disease in Man. Human infection with EMC virus has been observed on a number of occasions, and there is also serological evidence of infection in both man and lower animals. Soon after its isolation, a laboratory worker was infected with Mengo virus to give a meningoencephalitis of short duration, followed by rapid recovery. The virus was also isolated from four children in Germany who had indefinite illness with signs of meningeal involvement. An epidemic of mild febrile disease, called 3-day fever, occurred in Manila in 1945 and 1946 among American military personnel which serological evidence showed to be an infection with this virus. In these cases the febrile period lasted only 2 to 3 days; in some cases there were mild encephalitic symptoms, recovery was prompt and without sequelae, and there was no evidence of cardiac involvement.

The presence of neutralizing antibody

in both man and monkeys has been observed in serological surveys in Uganda and Tanganyika, with perhaps 3 to 4 per cent of human serums positive.[10] In this country antibody has been found in human serums with some frequency. Of one series of 300 serums examined, nine were found to contain antibody; of the nine positive serums, three were from convalescents from disease diagnosed as mild aseptic meningitis and five from patients diagnosed as having nonparalytic poliomyelitis.[216] An even higher incidence was reported in another group of serums; 21 per cent of serums from poliomyelitis convalescents and 16 per cent of "normal" serums were found to contain antibody to EMC virus.[97] Also in this country it has been found that 18 per cent of rat serums examined contained antibody.[216] The presence of the virus in South America has been shown by its isolation from a monkey, *Aotus trivirgatus*, in Colombia and the occurrence of antibody in man in Peru.[96] Monkeys may be infected by feeding mouse brain virus, but the evidence in general is taken to suggest that EMC virus occurs as an infection in rodents[109] which is transmissible to man and monkeys by mosquitoes and possibly in other ways as well.

RABIES

The rabies virus, together with poliovirus, is the most highly neurotropic of the viruses. All mammals, and some birds, are susceptible to infection, and in most species, but with important exceptions, it is practically uniformly fatal. The disease, also known as hydrophobia, has been known from ancient times in the Old World, in Egypt, Greece, Italy, and other parts of Europe, and the evidence suggests that it was imported into the Western Hemisphere from Europe. Prior to the eighteenth century, it was primarily a disease of wild animals, but in modern times the dog has assumed increasing importance and is responsible for the majority of cases of human infection with the rabies virus. The infectivity of saliva was demonstrated early in the nineteenth century; the virus was studied by Pasteur in the 1880's with the development of prophylactic treatment and in 1903 was shown by Remlinger to pass bacteria-proof filters.

Rabies Virus. The rabies virus is one of the larger of the neurotropic viruses and is 100 to 150 mμ in diameter. It is inactivated by the usual disinfectants, rapidly by formalin, but is relatively resistant to phenol, and is destroyed by ultraviolet irradiation. It is cultivable in the chick embryo, infected via the chorioallantoic membrane or yolk sac, or inoculated directly. In the infected embryo the virus may be found in the circulating blood as well as in the brain. It may be grown in mouse brain tissue culture, and, while it usually does not grow in cultures of nonnervous tissue, it has been cultivated in hamster kidney tissue culture, but without evidence of cytopathogenic changes.[105] The virus has been carried in suckling hamster kidney cell culture, demonstrable by the fluorescent antibody technique.[100] But in general, a tissue culture system showing cytopathic effects and producing virus to high titer has not yet been achieved. The virus has been partially purified from ground tissue supernatants by methods such as iso-electric precipitation, and fractionation with ammonium sulfate or alcohol precipitation in the cold, and behaves as if it were a globulin.

The virus appears to be a single antigenic type or a group of closely related types, but small immunological differences between the usual canine strains and the virus causing paralytic disease in cattle and transmitted by bats are demonstrable by the neutralization test. There are also strain differences in ability to invade the salivary glands.

Pathogenicity.[173] The neurotropism of the rabies virus is evident in the pathogenesis of the disease. From the site of the local injury where it is introduced into the tissues, the virus passes along the nerves to the central nervous system; viremia is uncommon, but hem-

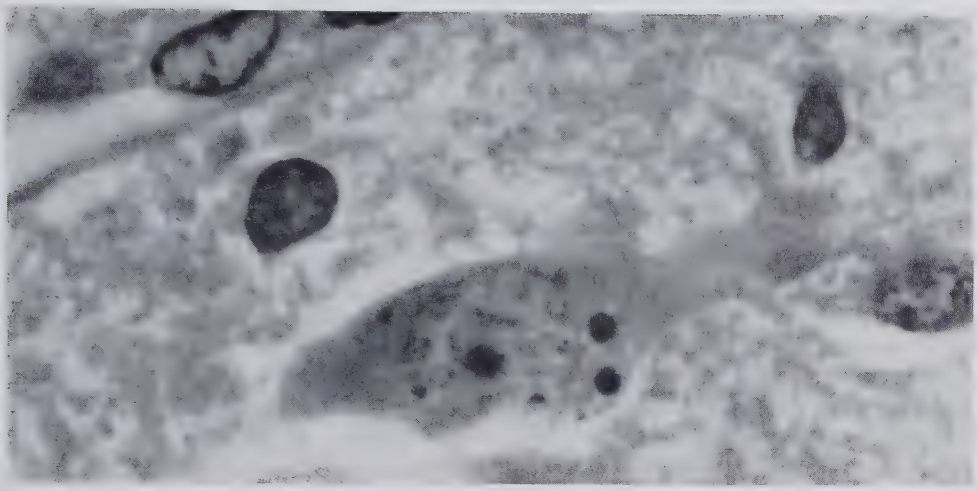


Figure 302. Negri bodies in a nerve cell in a section of the brain of a rabid cat. The three dark spherical bodies within the cytoplasm of the cell are Negri bodies. (Schleifstein.)

atogenous spread may occur under experimental conditions such as infection of the dog by the intravenous route. The significance of the neural pathway is indicated by the experimental infection of nerves, to produce infection first in the connected part of the spinal cord, and by the prevention of spread by resection of the nerve, the resected portion remaining infective. There is also evidence that, following its introduction directly into the brain, the virus may spread centrifugally, appearing in nerves such as the brachial and sciatic, producing interstitial neuritis. It has been suggested that the virus reaches the salivary glands in a similar manner.

Infection results in extensive destruction in the cerebral and cerebellar cortex, in the midbrain, basal ganglia, pons, and medulla, with neuronal degeneration and demyelinization. Similar changes are observed in the spinal cord and are most marked in the posterior horns. There is general hyperemia, mononuclear infiltration, and there may be small perivascular hemorrhages. The extent of these changes is related to the duration of the disease, and when death occurs soon, they may be minimal.

Negri bodies. The acidophilic inclusion body, the Negri body, is pathognomonic of rabies. It is found within the cytoplasm of the large ganglion cells, most abundantly in Ammon's horn, in the pyramidal cell layer of the cerebral cortex and the Purkinje cell layer of the cerebellum, and in the cranial nerve nuclei and in the basal ganglia. They are round in shape when lying free in the cytoplasm, may be compressed to an oval form by the nucleus, and are elongated when present in the dendrons. There is a considerable range in size of the Negri body, commonly given as 2 to 10 μ, but both smaller and larger bodies may be found.

When stained by Giemsa, the Negri body consists of a light blue ground substance containing red to red-violet basophilic granules. Sellers' stain, basic fuchsin and methylene blue in methanol, is regarded as superior by some workers, and stains the Negri body bright red and the internal granules are blue. Cytochemical studies have shown that the internal granules of the inclusion body contain DNA, and RNA is present in the matrix.[221] The Negri body is stained specifically by the fluorescent antibody technique which also stains the small eosinophilic inclusion bodies which are not ordinarily considered to be specific.[64]

Negri bodies are not invariably present in infections with canine, or street, virus and cannot be demonstrated in approximately 10 per cent, and at times as much as 25 per cent, of infections even though the virus may be isolated. They are not produced, at least in not

what is considered to be characteristic size, in infections with the rabbit-adapted, or fixed, virus.

Infection in Lower Animals. As indicated above, rabies virus occurs in reservoirs of infection in lower animals, most often carnivorous mammals. While practically any mammal, including herbivorous animals, may be infected at one time or another, the kinds of animals providing the reservoir of infection differ from one part of the world to another. The infection is endemic in foxes in Western Europe, and in the wolf in Eastern Europe, and may assume epizootic form from time to time in these animals. In West Africa dogs, both wild and domestic, are commonly infected, and the canine disease is called *oulou fato* locally, but the epidemiological behavior of the disease suggests that the canine infection is secondary to a reservoir of infection in some small rodent. In South Africa the infection is endemic in small carnivora, the meerkat and related animals, and the mongoose may contribute significantly to maintenance of the infection. In India jackals and wild dogs, and probably the mongoose to a lesser extent, are the important mammalian hosts of the virus.

In North America, Canada is relatively free from rabies,[52] but in the southern part of the continent the disease is endemic, and occasionally gives rise to epizootics, in foxes in the eastern and southeastern parts of the United States, and in skunks and other small rodents such as squirrels, raccoons, opossums, and muskrats in the Middle West and West, and coyotes in the West, and larger animals, such as deer may be infected also. In addition to these reservoirs of infection, rabies is also present in bats (see below).

The infection in wild animals may be regarded as the natural form of the disease. It serves as the source of infection for the domestic dog to give an urban type of the disease, the form most important to man.

Rabies in dogs. The infection in dogs is closely similar to that in wild animals and also to the human disease, and may be considered as representative. Dogs are infected by wild animals, or by other dogs, by the bite of the infected animal. Disease is not inevitable, and an appreciable portion of dogs bitten by a rabid animal, more than half, do not develop the disease. The usual incubation period is 3 to 10 weeks, but may be as short as 10 days, and under experimental conditions is dependent upon the amount of virus inoculated. The virus is present in the saliva prior to the appearance of characteristic clinical signs of the disease, and the animal is infectious during the last 3 to 5 days of the incubation period.

The disease takes two forms, the one furious or mad rabies and the other dumb rabies, depending upon the prolongation of the excitation period. At first the animal becomes restless and apprehensive and may become apathetic or seek out companionship. It then becomes hyperexcitable, responding to minimal stimuli, and runs about snapping and biting without discrimination and apparently oblivious of its surroundings. Beginning paralysis results in drooling of saliva due to inability to swallow, the gait may become abnormal, and the vocal cords are affected to give an eerie quality to the bark or howl. In dumb rabies the phase of excitation is relatively short, the animal appears to be profoundly depressed and unaware of its surroundings, overt paralysis becomes increasingly evident, and in general the more prolonged the disease the greater the paralysis. In either case the disease is practically invariably fatal within 10 days after onset of symptoms, although rare instances of recovery from the paralytic disease have been described. Occasionally death from rabies may occur suddenly without overt symptoms of disease.

On autopsy virus is present in the brain and cord, in the salivary and lacrimal glands, and in the kidney and pancreas, but seldom in the blood, and in general is not found in tissues of mesodermal origin. As noted earlier, strains of virus differ somewhat in their tissue affinities, some strains infecting

and multiplying in the salivary glands to a greater extent than others.

The clinical features of the disease are characteristic, and the canine infection is diagnosed by the presence of Negri bodies, but not eliminated by their absence, usually in impression smears of tissue from Ammon's horn and/or by isolation of the virus, commonly by intracerebral inoculation of mice. The diagnosis of canine rabies is of practical significance in relation to the institution of prophylactic inoculation in man exposed to infection by the bite of a dog.

Rabies in bats.[49] The occurrence of bat rabies, or chiropteran rabies, was first recognized in Brazil in 1908. The infection has since been found to be widely distributed in vampire bats, of which the most important species in this connection is *Desmodus rotundus murinus*, not only in South America, but also in Central America and Mexico. Since 1953 the infection has been found in a number of species of non-hemophagous bats in various parts of the United States.

The virus found in bats, and in animals infected by bats, differs from the canine strains in that it produces an ascending myelitis to give a spreading type of paralysis which is almost invariably fatal in animals other than bats. In cattle the hind limbs are paralyzed first, and the disease in man has at times been thought to be an infection with an aberrant type of poliovirus.

While the furious type of rabies is seen in both naturally and experimentally infected bats and is frequently fatal, infectious bats may survive for extended periods, as long as 3 months, and appreciable numbers survive to become healthy carriers of the virus. Bats are, therefore, an exception to the general rule that the infection is almost invariably fatal. The role of the brown fat in the bat infection has been emphasized by Sulkin. The virus has been isolated from this tissue taken from naturally infected bats[15] and Sulkin has shown[195] that the virus does in fact persist in this tissue, sometimes referred to as the hibernation gland, which functions as a "reservoiring mechanism" where little or no multiplication occurs during hibernation, but on warming the virus multiplies to reach detectable levels in other tissues.

The vampire bat bites at night to transmit the infection to cattle and other domestic animals and to man. The fatal paralytic disease in cattle has frequently assumed epizootic proportions and is known locally as *mal de caderas* in Brazil, *rabia parisiante* in Argentina, and *renguera, tronchado* or *derriengue* in Central America and Mexico. Within affected areas the disease has occurred in from 20 to as much as 50 per cent of cattle and other domestic animals. During 1929 to 1931 in Trinidad over 1000 cases occurred each year, and there were thirteen cases of fatal human disease in 1929 and four the following year.

Non-hemophagous insectivorous and fruit-eating bats in the United States have been found infected in fifteen states, ranging from a number of southern states to areas as distant as Montana, Minnesota, Ohio, New York, and Delaware. Rabies virus has been isolated from species of Dasypterus, Lasiurus, Myotis, Tadarida, Pipistrellus, Eptesicus, and Chilonycteris. Of these, the Mexican free-tailed bat, *Tadarida mexicana*, appears to be relatively heavily infected in Texas, as indicated by an incidence of neutralizing antibody in 16 to 70 per cent, varying with location, of specimens examined. In a detailed study carried out in Florida,[50] a total of 5503 colonial and free-living bats were examined, and the incidence of infection was found to be 0.2 per cent in the colonial forms and 1.5 per cent in the free-living forms. There appears to be little doubt that bats constitute a significant reservoir of rabies infection in the Western Hemisphere.

Attenuation. Alteration of the virulence of rabies virus by serial animal passage is the classical example of attenuation of virulence of microorganisms by this method and was applied by Pasteur in his development of attenuated virus for prophylactic immunization. The canine virus, as encountered in nature,

was designated by him "street virus." On intracerebral inoculation into the rabbit, disease is produced after an incubation period of about 2 weeks, but as the infection is passed serially in this animal, the incubation period is progressively reduced, reaching 8 days after twenty to twenty-five passages, and a limit of 7 days after another twenty to twenty-five passages. This modified virus was called "fixed virus" by Pasteur.

Fixed virus is characterized by an enhanced neurotropism, and inability to produce Negri bodies in the infected animal, and a reduced ability to invade the salivary glands. Pasteur's original strain of fixed virus, together with many of its substrains, is still available and has gone through 2000 or more passages in the rabbit. These strains vary among themselves in their virulence, as indicated by differences in incubation period and ability to produce infection by peripheral routes of inoculation in various experimental animals, but in general neurotropism is sufficiently enhanced that they are avirulent for the rabbit, dog, and man by the subcutaneous route. Fixed virus may also be prepared in other animals such as the mouse, guinea pig, or monkey by serial intracerebral passage; on intracerebral passage in the mouse, for example, street virus loses its ability to produce Negri bodies after about twenty-five passages.

The virus may also be adapted to the chick and chick embryo by continuous passage. The best-known of these strains is the strain isolated by Johnson in 1939 from a human case in day-old chicks and known as the Flury (the patient's name) strain. It was passed 136 times in chicks without observed changes except a decrease in the incubation period from 30 to 6 days, and then was passed in chick embryos. After repeated passage in the embryo, its pathogenicity for rabbits and dogs by extraneural routes of inoculation had disappeared, and at the 174th embryo passage intracerebral pathogenicity for the adult mouse was lost, and both dogs and rabbits are resistant to intracerebral inoculation with this high egg passage, or HEP, strain.[116, 117] Such attenuated strains are of particular interest as immunizing agents.

Disease in Man. Human infection with rabies virus is acquired by the bite of an infected animal. The infectiousness of the animal is determined by the presence of virus in the saliva, and it may be present for a few days, probably not more than 5, prior to the onset of symptoms. This time element is significant for if the animal does not develop rabies within 10 days, the saliva was probably not infectious at the time of the bite. The incubation period of the human disease ranges from a minimum of 2 weeks, or possibly slightly less, to as long as 5 to 7 months and is related to the severity of the bite and the location and innervation of the muscle tissue involved. Not all persons bitten by a known rabid animal develop the disease. Whether or not infection occurs is influenced by factors such as the severity of the wound, the removal of saliva when the bite is through heavy clothing, etc., but among equally severely bitten persons less than half may develop the disease.

The clinical disease in man is similar to that in the dog. The prodromal symptoms include fever, headache, malaise, nausea, and anorexia, and there is anxiety or depression, and often sensations of itching, burning, or tingling at the site of the wound. The onset of the excitation phase is gradual and indicated by increasing nervousness and apprehension. The characteristic symptom is the painful spasmodic contraction of the muscles involved in swallowing and the accessory muscles of respiration when fluid comes in contact with the fauces, and subsequently suggestion of the act of swallowing may precipitate muscle spasm; this feature of the disease gives it the name hydrophobia. As the disease progresses there may be muscular contractions and tremor, choking, and resulting cyanosis; convulsions occur, and there is often maniacal behavior. Such periods of excitation may be interspersed with lucid intervals. Death usually occurs during convulsions. In some instances the patient survives the period

of excitement and becomes apathetic; stupor and coma follow, and an ascending type of paralysis develops. So far as is known the disease in man is invariably fatal.

The pathological changes in human rabies are similar to those seen in dogs and include some degree of hyperemia and severe neuronal degeneration in the midbrain, basal ganglia, and pons. Degenerative changes are marked in the nuclei of cranial nerves and in the thalamus and hypothalamus, and Negri bodies are present. Neuronal degeneration in the cord is most marked in the posterior horns, and when the bite has occurred on an extremity, the corresponding posterior horn shows extensive destruction with neuronophagia and may extend into the dorsal root ganglia.

Laboratory diagnosis.[209] The laboratory diagnosis of rabies is usually of primary importance in the animal which has bitten or otherwise exposed man. As indicated above, the saliva is infectious prior to the development of symptoms. Premature destruction of the animal is undesirable and if possible the disease should be allowed to develop if present to facilitate diagnosis. Such diagnosis, in lower animals or man, is based on the demonstration of Negri bodies, usually in impression smears of Ammon's horn. Giemsa or Sellers' stain may be used, and the fluorescent antibody technique allows the identification of smaller bodies not ordinarily identifiable as Negri bodies.[139] The technique may, in fact, be used to identify the virus in the salivary glands of infected animals.[65] The virus may also be isolated by intracerebral inoculation of mice and demonstration of Negri bodies in the mouse brain.

Immunity. Complement-fixing and neutralizing antibodies are formed in response to immunization with vaccine and in unimmunized individuals during the course of the disease. The immune response to infection is not an effective one in man, nor does antibody activity have diagnostic value except in animals such as bats which recover from the disease. Antibody titrations are useful, however, for assaying the response to immunizing preparations, identification of virus by neutralization tests, and other experimental purposes.

Prophylaxis. There seems to be no significant change in the antigenicity of rabies virus on adaptation to give fixed virus, and effective immunization to rabies in man and dogs is possible because of the long incubation period in the first instance which allows sufficient time to induce an effective immune response. Immunization of dogs on a mass basis makes possible the control of the urban type of the disease. The immune response to vaccines is not long lasting, and in the case of dogs must be repeated each year.

The extended incubation period in rabies makes possible the development of an effective active immunity even though the immunization is begun after exposure. Pasteur's original method was the inoculation of the exposed person daily with emulsions of fixed virus in rabbit spinal cord, beginning with cord which had been dried 14 days, and gradually increasing the amount of virus by the use of cord dried for shorter and shorter periods. Subsequently other methods, using gradually increasing doses of active virus or virus inactivated by various chemical and physical methods, have been used. In this country fixed virus in rabbit brain tissue, inactivated by phenol (Semple vaccine), formalin, etc., is given in daily subcutaneous inoculations for a period of 14 or 21 days,[70] and similar preparations are used for the immunization of dogs. Phenolized vaccines may be stabilized by lyophilization and still retain immunogenic potency.[214] All such vaccines contain relatively large amounts of nerve tissue, and there is reason to believe that complications, usually neuritis or myelitis with occasional permanent damage,[7] are in large part the consequence of an iso-immune response to organ-specific antigen (Chap. 13).

The HEP Flury strain, adapted to the chick embryo as described above, has been found to give a neutralizing antibody response when given in an active

form[163] and has also been grown in duck embryos and inactivated with β-propriolactone, the inactivated vaccine giving a neutralizing antibody response.[4, 178] Such preparations do not, of course, contain large amounts of nerve tissue.

Antiserum contains neutralizing antibody demonstrable experimentally and has been of interest as a prophylactic or an adjunct prophylactic for the human disease. It is evident that if antiserum containing neutralizing antibody is given very soon, within a few days, of exposure to infection, it gives an appreciable protective effect, and a combination of antiserum and vaccine has been found to give better protection than either alone.[9, 69, 70, 174]

REFERENCES

1. Anderson, C. R., Aitken, T. H. G., Spence, L. P., and Downs, W. G.: Kairi virus, a new virus from Trinidadian forest mosquitoes. Amer. J. Trop. Med. Hyg., 1960, **9:**70–72.
2. Anderson, C. R., Spence, L. P., Downs, W. G., and Aitken, T. H. G.: Manzanilla virus: A new virus isolated from the blood of a howler monkey in Trinidad, W. I. Amer. J. Trop. Med. Hyg., 1960, **9:**78–80.
3. Anderson, C. R., Spence, L., Downs, W. G., and Aitken, T. H. G.: Oropouche virus: A new human disease agent from Trinidad, West Indies. Amer. J. Trop. Med. Hyg., 1961, **10:**574–578.
4. Anderson, G. R., Schnurrenberger, P. R., Masterson, R. A., and Wentworth, F. H.: Avian embryo rabies immunization. I. Duck-embryo vaccine administered intradermally in man. Amer. J. Hyg., 1960, **71:**158–167.
5. Anderson, S. G., Dobrotworsky, N. V., and Stevenson, W. J.: Murray Valley encephalitis in the Murray Valley, 1956 and 1957. Med. J. Australia, 1958, **2:**15–17.
6. Anderson, S. G., Price, A. V. G., Nanadai-Koia, and Slater, K.: Murray Valley encephalitis in Papua and New Guinea. II. Serological survey. Med. J. Australia, 1960, **2:**410–413.
7. Applebaum, E., Greenberg, M., and Nelson, J.: Neurological complications following antirabies vaccination. J. Amer. Med. Assn., 1952, **151:**188–191.
8. Armstrong, C., and Lillie, R. D.: Experimental lymphocytic choriomeningitis of monkeys and mice produced by a virus encountered in studies of the 1933 St. Louis encephalitis epidemic. Pub. Health Repts., 1934, **49:**1019–1027.
9. Atanasiu, P., et al.: Rabies neutralizing antibody response to different schedules of serum and vaccine inoculations in non-exposed persons: Part 3. Bull. W. H. O., 1961, **25:**103–114.
10. Barski, G., and Cornefert, F.: Position particulière des encéphalomyélites de type Mengo par rapport aux poliomyélites. Répartition d'anticorps spécifiques chez certaines populations d'Afrique centrale et d'autres zones géographiques. Bull. W. H. O., 1957, **17:**991–999.
11. Beadle, L. D.: Status of mosquito-borne encephalitis in the United States. Pub. Health Repts., 1959, **74:**84–90.
12. Bearcroft, W. G. C.: The histopathology of the liver of yellow fever-infected rhesus monkeys. J. Path. Bact., 1957, **74:**295–303.
13. Bearcroft, W. G. C.: Cytological and cytochemical studies on the liver cells of yellow fever-infected rhesus monkeys. J. Path. Bact., 1960, **80:**19–31.
14. Bearcroft, W. G. C.: Electron-microscope studies on the liver cells of yellow-fever-infected rhesus monkeys. J. Path. Bact., 1960, **80:**421–426.
15. Bell, J. F., and Moore, G. J.: Rabies virus isolated from brown fat of naturally infected bats. Proc. Soc. Exptl. Biol. Med., 1960, **103:**140–142.
16. Benson, L. M., and Hotchin, J. E.: Cytopathogenicity and plaque formation with lymphocytic choriomeningitis virus. Proc. Soc. Exptl. Biol. Med., 1960, **103:**623–625.
17. Bhatt, P. N., and Work, T. H.: Tissue culture studies on arbor viruses of the Japanese B-West Nile complex. Proc. Soc. Exptl. Biol. Med., 1957, **96:**213–218.
18. Boshell, J.: Marche de la fièvre jaune selvatique vers les regiones du nord-ouest de l'Amérique centrale. Bull. W. H. O., 1957, **16:**431–436.
19. Boyd, J. S. K.: Yellow fever and its prevention. J. Roy. Inst. Pub. Health Hyg., 1959, **22:**11–25.
20. Brody, J. A., Burns, K. F., Browning, G., and Schattner, J. D.: Apparent and inapparent attack rates for St. Louis encephalitis in a selected population. New England J. Med., 1959, **261:**644–646.
21. Brown, L. V.: Color change in assay of WEE virus in tissue culture. Amer. J. Hyg., 1958, **67:**214–236.
22. Burgdorfer, W.: Colorado tick fever. The behavior of CTF virus in the porcupine. J. Infect. Dis., 1959, **104:**101–104.
23. Burgdorfer, W.: Colorado tick fever. II. The behavior of Colorado tick fever virus in rodents. J. Infect. Dis., 1960, **107:**384–388.
24. Burgdorfer, W., and Eklund, C. M.: Studies on the ecology of Colorado tick fever virus in western Montana. Amer. J. Hyg., 1959, **69:**127–137.
25. Burgdorfer, W., and Eklund, C. M.: Colorado tick fever. I. Further ecological studies in western Montana. J. Infect. Dis., 1960, **107:**379–383.
26. Bustamante, M. E.: La fiebre amarilla en México y su origen en América. Instituto de Salubridad y Enfermendades Tropicales, Mexico. 1958.
27. Casals, J.: Immunological relationships among nervous system viruses. J. Exptl. Med., 1944, **79:**341–359.
28. Casals, J.: Viruses: The versatile parasites. I. The arthropod-borne group of animal viruses. Trans. N. Y. Acad. Sci., Ser. 2, 1957, **19:**219–235.
29. Casals, J.: Antigenic relationship between

Powassan and Russian spring-summer encephalitis viruses. Canadian Med. Assn. J., 1960, **82:**355–358.
30. Casals, J.: Procedures for identification of arthropod-borne viruses. Bull. W. H. O., 1961, **24:**723–734.
31. Casals, J., and Whitman, L.: Mayaro virus: A new human disease agent. I. Relationship to other arbor viruses. Amer. J. Trop. Med. Hyg., 1957, **6:**1004–1011.
32. Casals, J., and Whitman, L.: A new antigenic group of arthropod-borne viruses. The Bunyamwera group. Amer. J. Trop. Med. Hyg., 1960, **9:**73–77.
33. Casals, J., and Whitman, L.: Group C, a new serological group of hitherto undescribed arthropod-borne viruses. Immunological studies. Amer. J. Trop. Med. Hyg., 1961, **10:**250–258.
34. Chamberlain, R. W.: Vector relationships of the arthropod-borne encephalitides in North America. Ann. N. Y. Acad. Sci., 1958, **70:** 312–319.
35. Chang, T. W., and Weinstein, L.: Characteristics of growth of encephalomyocarditis virus in cultures of mouse muscle. Proc. Soc. Exptl. Biol. Med., 1959, **102:**181–183.
36. Cheng, P.-Y.: Purification, size and morphology of a mosquito-borne animal virus, Semliki forest virus. Virology, 1961, **14:**124–131.
37. Cheng, P.-Y.: Some physical properties of hemagglutinating and complement-fixing particles of Semliki forest virus. Virology, 1961, **14:**132–140.
38. Clarke, D. H., and Casals, J.: Techniques for hemagglutination and hemagglutination-inhibition with arthropod-borne viruses. Amer. J. Trop. Med. Hyg., 1958, **7:**561–573.
39. Collier, W. A., and De Wit, M.: Immunization experiments in mice with dead or neutralized yellow fever virus. Trop. Geograph. Med., 1960, **12:**258–262.
40. De Groot, C. J., Metzger, J. F., Smith, C. W., and Hoggan, M. D.: Demonstration of yellow fever virus in human cell culture by immunofluorescence. Virology, 1960, **12:**317–320.
41. Dick, G. W. A.: Epidemiological notes on some viruses isolated in Uganda (yellow fever, Rift Valley fever, Bwamba fever, West Nile, Mengo, Semliki forest, Bunyamwera, Ntaya, Uganda S and Zika viruses). Trans. Roy. Soc. Trop. Med. Hyg., 1953, **47:**13–48.
42. Dick, G. W. A., and Haddow, A. J.: Uganda S virus: Hitherto unrecorded virus isolated from mosquitoes in Uganda; isolation and pathogenicity. Trans. Roy. Soc. Trop. Med. Hyg., 1952, **46:**600–618.
43. Dick, G. W. A., Kitchen, S. F., and Haddow, A. J.: Zika virus: Isolations and serological specificity. Trans. Roy. Soc. Trop. Med. Hyg., 1952, **46:**509–520.
44. Downs, W. G., Spence, L., Aitken, T. H. G., and Whitman, L.: Cache Valley virus, isolated from a Trinidadian mosquito, *Aedes scapularis*. West Indian Med. J., 1961, **10:**13–15.
45. Durand, P.: Virus filtrant pathogène pour l'homme et les animaux de laboratoire et à affinités meningée et pulmonaire. Arch. Inst. Pasteur Tunis, 1940, **29:**179–227.
46. Eklund, C. M., Kohls, G. M., and Brennan, J. M.: Distribution of Colorado tick fever and virus-carrying ticks. J. Amer. Med. Assn., 1955, **157:**335–337.
47. Eklund, C. M., Kohls, G. M., and Jellison, W. O.: Isolation of Colorado tick fever virus from rodents in Colorado. Science, 1958, **128:**413.
48. Elton, N. W.: Yellow fever in Central America: The imminent threat to Mexico and the United States. Amer. J. Pub. Health, 1956, **46:**1259–1265.
49. Enright, J. B.: Bats, and their relation to rabies. Ann. Rev. Microbiol., 1956, **10:**369–392.
50. Enright, J. B.: Geographical distribution of bat rabies in the United States, 1953–1960. Amer. J. Pub. Health, 1962, **52:**484–488.
51. Feemster, R. F.: Equine encephalitis in Massachusetts. New England J. Med., 1957, **257:** 701–704.
52. Fenje, P., and Amies, C. R.: The problem of rabies in Canada. Canadian Med. Assn. J., 1960, **82:**243–245.
53. Findlay, G. M.: Durand's disease; virus infection transmissible to animals and man. Trans. Roy. Soc. Trop. Med. Hyg., 1942, **35:**303–318.
54. French, E. L., Anderson, S. G., Price, A. V. G., and Rhodes, F. A.: Murray Valley encephalitis in New Guinea. I. Isolation of Murray Valley encephalitis virus from the brain of a fatal case of encephalitis occurring in a Papuan native. Amer. J. Trop. Med. Hyg., 1957, **6:**827–834.
55. Frothingham, T. E.: Tissue culture applied to the study of Sindbis virus. Amer. J. Trop. Med. Hyg., 1955, **4:**863–871.
56. Gajdusek, D. C., Anslow, R. O., Hubbell, E. J., and Yager, R. H.: Tissue culture studies of Venezuelan equine encephalomyelitis. I. Propagation in human uterine tissue. J. Immunol., 1954, **72:**224–228.
57. Galindo, P., and De Rodaniche, E.: Birds as hosts of Ilheus virus in Panama. Amer. J. Trop. Med. Hyg., 1961, **10:**395–396.
58. Gauld, R. L., and Craig, J. P.: Epidemiological pattern of localized outbreaks of epidemic hemorrhagic fever. Amer. J. Hyg., 1954, **59:** 32–38.
59. Gebhardt, L. P., and Hill, D. W.: Overwintering of western equine encephalomyelitis virus. Proc. Soc. Exptl. Biol. Med., 1960, **104:**695–698.
60. Gerloff, R. K., and Eklund, C. M.: A tissue culture neutralization test for Colorado tick fever antibody and use of the test for serologic surveys. J. Infect. Dis., 1959, **104:**174–183.
61. Gerloff, R. K., and Larson, C. L.: Experimental infection of rhesus monkeys with Colorado tick fever virus. Amer. J. Path., 1959, **35:** 1043–1054.
62. Gildemeister, H., et al.: Praktikum der Virusdiagnostik. Gustav Fischer Verlag, Stuttgart. 1961.
63. Goldblum, N., Sterk, V. V., and Jasinska-Klingberg, W.: The natural history of West Nile fever. II. Virological findings and the development of homologous and heterologous antibodies. Amer. J. Hyg., 1957, **66:** 363–380.
64. Goldwasser, R. A., and Kissling, R. E.: Fluorescent antibody staining of street and fixed rabies virus antigen. Proc. Soc. Exptl. Biol. Med., 1958, **98:**219–223.

65. Goldwasser, R. A., Kissling, R. E., Carski, T. R., and Hosty, T. S.: Fluorescent antibody staining of rabies virus antigens in the salivary glands of rabid animals. Bull. W. H. O., 1959, **20:**579–588.
66. Gomes, G., and Causey, O. R.: Bussuquara, a new arthropod-borne virus. Proc. Soc. Exptl. Biol. Med., 1959, **101:**275–279.
67. Gordon Smith, C. E.. and Holt, D.: Chromatography of arthropod-borne viruses on calcium phosphate columns. Bull. W. H. O., 1961, **24:**749–759.
68. Groot, H., Oya, A., Bernal, C., and Baretto-Reyes, P.: Guaroa virus, a new agent isolated in Columbia, South America. Amer. J. Trop. Med. Hyg., 1959, **8:**604–609.
69. Habel, K.: Antiserum in the prophylaxis of rabies. Bull. W. H. O., 1954, **10:**781–788.
70. Habel, K.: Rabies prophylaxis in man. Pub. Health Repts., 1957, **72:**667–673.
71. Hallauer, C.: Züchtung von Gelbfiebervirus in menschlichen Explantaten. Arch. f. d. gesamte Virusf., 1959, **9:**428–441.
72. Halstead, S. B., and Buescher, E. L.: Hemorrhagic disease in rodents associated with Thai hemorrhagic fever. Science, 1961, **134:** 475–476.
73. Hammon, W. McD., Rees, D. M., Casals, J., and Meikeljohn, G.: Experimental transmission of Japanese B encephalitis virus by *Culex tritaeniorhynchus* and *Culex pipiens* var. *pallens,* suspected natural vectors. Amer. J. Hyg., 1949, **50:**46–50.
74. Hammon, W. McD., and Reeves, W. C.: California encephalitis virus: Newly described agent. California Med., 1952, **77:**303–309.
75. Hammon, W. McD., Reeves, W. C., and Sather, G.: California encephalitis, a newly described agent. II. Isolation and attempts to identify and characterize the agent. J. Immunol., 1952, **69:**493–510.
76. Hammon, W. McD., Rudnick, A., and Sather, G. E.: Viruses associated with epidemic hemorrhagic fevers of the Philippines and Thailand. Science, 1960, **131:**1102–1103.
77. Hammon, W. McD., Sather, G. E., and McClure, H. E.: Serologic survey of Japanese B encephalitis virus infection in birds in Japan. Amer. J. Hyg., 1958, **67:**118–133.
78. Hammon, W. McD., Tiggertt, W. D., Sather, G., and Schenker, H.: Isolations of Japanese B encephalitis virus from naturally infected *Culex tritaeniorhynchus* collected in Japan. Amer. J. Hyg., 1949, **50:**51–56.
79. Hannoun, C., and Panthier, R.: Application de méthode des plages en cultures cellulaires à l'étude du virus-vaccin amaril 17D. Bull. Soc. Path. Exot., 1960, **53:**424–430.
80. Havens, W. P.: Hepatitis, yellow fever and dengue. Ann. Rev. Microbiol., 1954, **8:**289–310.
81. Havlik, O., Kolman, J. M., and Lim, D.: The incidence of tick-borne encephalitis in wild birds. J. Hyg. Epidemiol. Microbiol. Immunol., 1957, **1:**367–376.
82. Hayes, R. O., Daniels, J. B., and MacCready, R. A.: Western encephalitis virus in Massachusetts. Proc. Soc. Exptl. Biol. Med., 1961, **108:**805–808.
83. Hearn, H. J., Jr.: A variant of Venezuelan equine encephalomyelitis virus attenuated for mice and monkeys. J. Immunol., 1960, **84:** 626–629.
84. Henderson, J. R., and Taylor, R. M.: Propagation of certain arthropod-borne viruses in avian and primate cell cultures. J. Immunol., 1960, **84:**590–598.
85. Henderson, J. R., and Taylor, R. M.: Phlebotomus (sandfly) fever viruses in tissue culture. Amer. J. Trop. Med. Hyg., 1960, **9:**32–36.
86. Hess, A. D., and Holden, P.: The natural history of the arthropod-borne encephalitides in the United States. Ann. N. Y. Acad. Sci., 1958, **70:**294–311.
87. Heymann, C. S., Kokernot, R. H., and De Meillon, B.: Wesselsbron virus infections in man. South African Med. J., 1958, **32:**543–545.
88. Holden, P., and Hess, A. D.: Cache Valley virus, a previously undescribed mosquito-borne agent. Science, 1959, **130:**1187.
89. Hotta, S.: Propagation of dengue virus in tissue culture. Acta Tropica, 1959, **16:**108–150.
90. Hotta, S., and Evans, C. A.: Cultivation of mouse-adapted dengue virus (type 1) in rhesus monkey tissue culture. J. Infect. Dis., 1956, **98:**88–97.
91. Hotta, S., and Evans, C. A.: Cultivation of type 2 dengue virus in rhesus kidney tissue culture. Proc. Soc. Exptl. Biol. Med., 1956, **93:** 153–155.
92. Hughes, J. H., and Porter, J. E.: Measures against yellow fever entry into the United States. Pub. Health Repts., 1958, **73:**1101–1106.
93. Hughes, T. P., and Perlowagora, A.: The antigenic relationships of certain viruses capable of producing encephalitis in mice as shown by complement fixation tests. J. Immunol., 1950, **65:**155–161.
94. Imam, I. Z. E., and Hammon, W. McD.: Challenge of hamsters with Japanese B, St. Louis and Murray Valley encephalitis viruses after immunization by West Nile infection plus specific vaccine. J. Immunol., 1957, **79:**243–252.
95. Iyer, C. G. S., et al.: Kyasanur Forest disease. Part VII. Pathological findings in monkeys, *Presbytis entellus* and *Macaca radiata,* found dead in the forest. Indian J. Med. Res., 1960, **48:**276–286.
96. Jonkers, A. H.: Serosurvey of encephalomyocarditis virus neutralizing antibodies in southern Louisiana and Peruvian Indian populations. Amer. J. Trop. Med. Hyg., 1961, **10:**593–598.
97. Jungeblutt, C. W.: Neutralization of Columbia-SK and Yale-SK virus by polio-convalescent and normal human sera. Arch. Pediatrics, 1950, **67:**519–530.
98. Jungherr, E. L., Helmbolt, C. F., Satriano, S. F., and Luginbuhl, R. E.: Investigation of eastern equine encephalomyelitis. III. Pathology in pheasants and incidental observations in feral birds. Amer. J. Hyg., 1958, **67:**10–20.
99. Jungherr, E. L., and Wallis, R. C.: Investigation of eastern equine encephalomyelitis. I. General aspects. Amer. J. Hyg., 1958, **67:** 1–3.
100. Kaplan, M. M., Forsek, Z., and Koprowski, H.: Demonstration of rabies virus in tissue culture with fluorescent antibody technique. Bull. W. H. O., 1960, **22:**434–435.
101. Kerr, J. A.: Studies on certain viruses isolated in tropics of Africa and South America. Immunological reactions as determined by

cross complement-fixation tests. J. Immunol., 1952, **68:**461–472.
102. Kilham, L., Mason, P., and Davies, J. N. P.: Pathogenesis of fatal encephalomyocarditis (EMC) virus infections in albino rats. Proc. Soc. Exptl. Biol. Med., 1955, **90:**383–387.
103. Kissling, R. E.: Growth of several arthropod-borne viruses in tissue culture. Proc. Soc. Exptl. Biol. Med., 1957, **96:**290–294.
104. Kissling, R. E.: Host relationship of some encephalitides. Ann. N. Y. Acad. Sci., 1958, **70:**320–327.
105. Kissling, R. E.: Growth of rabies virus in non-nervous tissue. Proc. Soc. Exptl. Biol. Med., 1958, **98:**223–225.
106. Kissling, R. E.: The arthropod-borne viruses of man and other animals. Ann. Rev. Microbiol., 1960, **14:**261–282.
107. Kissling, R. E., Chamberlain, R. W., Sudia, W. D., and Stamm, D. D.: Western equine encephalomyelitis in wild birds. Amer. J. Hyg.. 1957, **66:**48–55.
108. Kissling, R. E., Stamm, D. D., Chamberlain, R. W., and Sudia, W. D.: Birds as winter hosts for eastern and western equine encephalomyelitis viruses. Amer. J. Hyg., 1957, **66:**42–47.
109. Kissling, R. E., Vanella, J. M., and Schaeffer, M.: Recent isolations of encephalomyocarditis virus. Proc. Soc. Exptl. Biol. Med., 1956, **91:**148–150.
110. Kokernot, R. H., et al.: Middelburg virus, a hitherto unknown agent isolated from Aedes mosquitoes during an epizootic in sheep in the Eastern Cape Province. South African J. Med. Sci., 1957, **22:**145–153.
111. Kokernot, R. H., McIntosh, B. M., and Worth, C. B.: Ndumu virus, a hitherto unknown agent, isolated from culicine mosquitoes collected in Northern Natal, Union of South Africa. Amer. J. Trop. Med. Hyg., 1961, **10:**383–386.
112. Kokernot, R. H., Smithburn, K. C., De Meillon, B., and Paterson, H. E.: Isolation of Bunyamwera virus from a naturally infected human being and further isolations from *Aedes* (*Banksinella*) *circumluteolus* Theo. Amer. J. Trop. Med. Hyg., 1958, **7:**579–584.
113. Kokernot, R. H., Smithburn, K. C., Muspratt, J., and Hodgson, B.: Spondweni virus, an agent previously unknown, isolated from *Taeniorhynchus* (*Mansonioides*) *uniformis* Theo. South African J. Med. Sci., 1957, **22:** 103–112.
114. Kokernot, R. H., Smithburn, K. C., Paterson, H. E., and McIntosh, B. M.: Isolation of Germiston virus, a hitherto unknown agent, from culicine mosquitoes, and a report of infection in two laboratory workers. Amer. J. Trop. Med. Hyg., 1960, **9:**62–69.
115. Kokernot, R. H., Smithburn, K. C., Weinbren, M. P., and De Meillon, B.: Isolation of Pongola virus from *Aedes* (*Banksinella*) *circumluteolus* Theo. South African J. Med. Sci., 1957, **22:**81–92.
116. Koprowski, H.: Biological modification of rabies virus as a result of its adaptation to chicks and developing chick embryos. Bull. W. H. O., 1954, **10:**709–724.
117. Koprowski, H., Black, J., and Nelsen, D. J.: Studies on chick-embryo-adapted rabies virus. VI. Further changes in pathogenic properties following prolonged cultivation in developing chick embryo. J. Immunol., 1954, **72:**94–106.
118. Laemert, H. W., Jr., and Hughes, T. P.: The virus of Ilheus encephalitis; isolation, serological specificity and transmission. J. Immunol., 1947, **55:**61–67.
119. La Motte, L. C., Jr.: Japanese B encephalitis in bats during simulated hibernation. Amer. J. Hyg., 1958, **67:**101–108.
120. Lee, H. W., Hinz, R. W., and Scherer, W. F.: Porcine kidney cell cultures for propagation and assay of Japanese encephalitis virus. Proc. Soc. Exptl. Biol. Med., 1958, **99:**579–583.
121. Lennette, E. H., et al.: Turlock virus: A presumably new arthropod-borne virus. Isolation and identification. Amer. J. Trop. Med. Hyg., 1957, **6:**1024–1035.
122. Lennette, E. H., Ota, M. I., and Hoffman, M. N.: Turlock virus: A presumably new arthropod-borne virus. A description of some of its properties.. Amer. J. Trop. Med. Hyg., 1957, **6:**1036–1046.
123. Lepine, P., Mollaret, P., and Kreis, B.: Réceptivité de l'homme au virus murin de la chorioméningite lymphocytaire. Reproduction expérimentale de la méningite lymphocytaire bénigne. C. R. Acad. Sci., 1937, **204:** 1846–1848.
124. Levkovich, E. N.: Present status of problems of epidemic encephalitides. Problems Virol., 1957, **2:**133–145. (Translated from the Russian.)
125. Levkovich, E. N., and Karpovich, L. G.: Comparative study of the viruses of the tick-borne encephalitis group in HeLa cell cultures. Problems Virol., 1960, **5:**31–40. (Translated from the Russian.)
126. Levkovich, E. N., and Pogodina, V. V.: The alimentary route of infection in tick-borne encephalitis. Problems Virol., 1958, **3:**154–160. (Translated from the Russian.)
127. Likar, M., and Dane, D. S.: An illness resembling acute poliomyelitis caused by a virus of the Russian spring-summer encephalitis/louping ill group in Northern Ireland. Lancet, 1958, **i:**456–458.
128. Lowenthal, J. P., Berman, S., and Grogan, E. W.: Eastern equine encephalomyelitis vaccine prepared in cell culture. Science, 1961, **134:**565–566.
129. Luginbuhl, R. E., et al.: Investigation of eastern equine encephalomyelitis. II. Outbreaks in Connecticut pheasants. Amer. J. Hyg., 1958, **67:**4–9.
130. MacLeod, C. M., et al.: United States medical mission on microbiology and epidemiology to Soviet Union. J. Amer. Med. Assn., 1956, **162:**656–658.
131. Marberg, K., et al.: The natural history of West Nile fever. I. Clinical observations during an epidemic in Israel. Amer. J. Hyg., 1956, **64:**259–269.
132. Mayer, C. F.: Epidemic hemorrhagic fever of Far East, or endemic hemorrhagic nephrosonephritis; short outline of the disease, with supplemental data on results of experimental inoculation of human volunteers. Military Surgeon, 1952, **110:**276–284.
133. McIntosh, B. M., Kokernot, R. H., and Paterson, H. E.: Witwatersrand virus: An apparently new virus isolated from culicine mosquitoes. South African J. Med. Sci., 1960, **25:**33–37.

134. McIntosh, B. M., Kokernot, R. H., Paterson, H. E., and De Meillon, B.: Isolation of Spondweni virus from four species of culicine mosquitoes and a report of two laboratory infections with the virus. South African Med. J., 1961, **35:**647–650.
135. McLean, D. M.: Vectors of Murray Valley encephalitis. J. Infect. Dis., 1957, **100:**223–227.
136. McLean, D. M., et al.: Powassan virus: Investigations of possible natural cycles of infection. J. Infect. Dis., 1961, **109:**19–23.
137. McLean, D. M., and Donohue, W. L.: Powassan virus: Isolation of virus from a fatal case of encephalitis. Canadian Med. Assn. J., 1959, **80:**708–711.
138. McLean, D. M., MacPherson, L. W., Walker, S. J., and Funk, G.: Powassan virus: Surveys of human and animal sera. Amer. J. Pub. Health, 1960, **50:**1539–1544.
139. McQueen J. L., Lewis, A. L., and Schneider, N. J.: Rabies diagnosis by fluorescent antibody. I. Its evaluation in a public health laboratory. Amer. J. Pub. Health, 1960, **50:** 1743–1752.
140. Medearis, D. N., Jr., and Kilbrick, S.: An evaluation of various tissue cultures for isolation of eastern equine encephalomyelitis virus. Proc. Soc. Eptl. Biol. Med., 1958, **97:** 152–158.
141. Melnick, J. L., et al.: Isolation from human sera in Egypt of a virus apparently identical to West Nile virus. Proc. Soc. Exptl. Biol. Med., 1951, **77:**661–665.
142. Mettler, N., Buckley, S. M., and Casals, J.: Propagation of Junin virus, the etiological agent of Argentinian hemorrhagic fever, in HeLa cell cultures. Proc. Soc. Exptl. Biol. Med., 1961, **107:**684–688.
143. Miles, J. A. R.: Epidemiology of the arthropod-borne encephalitides. Bull. W. H. O., 1960, **22:**339–371.
144. Miles, J. A. R., and Howes, D. W.: Encephalitis virus isolated in South Australia; serological observations on virus and its relation to other arthropod-borne encephalitides. Australian J. Exptl. Biol. Med. Sci., 1952, **30:**353–362.
145. Miller, J. K., Tompkins, V. N., and Sieracki, J. C.: Pathology of Colorado tick fever in experimental animals. Arch. Path., 1961, **72:** 149–157.
146. Mundel, B., and Gear, J.: Rift Valley fever. I. The occurrence of human cases in Johannesburg. South African Med. J., 1951, **25:** 797–800.
147. Murnane, T. G., Craighead, J. E., Mondragon, H., and Shelokov, A.: Fatal disease of swine due to encephalomyocarditis virus. Science, 1961, **131:**498–499.
148. Narashimha Murthy, D. P.: New virus disease —Kyasanur Forest disease. J. Indian Med. Assn., 1958, **31:**125–127.
149. Okuno, T.: Immunological studies relating two recently isolated viruses, Germiston virus from South Africa and Ilesha virus from West Africa, to the Bunyamwera group. Amer. J. Trop. Med. Hyg., 1961, **10:**223–226.
150. Panthier, R., Bres, P., Husson, R. A., and Hannoun, C.: Étude des anticorps sériques provoqués par la vaccination antimarile. Bull. Soc. Path. Exot., 1958, **51:**589–600.
151. Peller, S.: Walter Reed, C. Finlay and their predecessors around 1800. Bull. Hist. Med., 1959, **33:**195–211.
152. Pickens, E. G., and Luoto, L.: Tissue culture studies with Colorado tick fever virus. I. Isolation and propagation in KB cultures. J. Infect. Dis., 1958, **103:**102–107.
153. Pogodina, V. V.: Latent inmmunisation of the population in foci of tick-borne encephalitis by means of fresh goat's milk. Problems Virol., 1959, **4:**43–48. (Translated from the Russian.)
154. Porterfield, J. S.: A plaque technique for the titration of yellow fever virus and antisera. Trans. Roy. Soc. Trop. Med. Hyg., 1959, **53:**458–466.
155. Porterfield, J. S.: The nature of serological relationships among arthropod-borne viruses. Adv. Virus Res., 1962, **9:**127–156.
156. Prapont Pityaratn: Pathology of Thailand epidemic hemorrhagic fever. Amer. J. Trop. Med. Hyg., 1961, **10:**767–772.
157. Reeves, W. C.: Overwintering of arthropod-borne viruses. Progress Med. Virol., 1961, **3:**59–78.
158. Reeves, W. C., and Hammon, W. McD.: California encephalitis, a newly described agent. III. Mosquito infection and transmission. J. Immunol., 1952, **69:**511–514.
159. Reeves, W. C., Hutson, G. A., Bellamy, R. E., and Scrivani, R. P.: Chronic latent infections of birds with western equine encephalomyelitis virus. Proc. Soc. Exptl. Biol. Med., 1958, **97:**733–736.
160. Roca-García, M.: The isolation of three neurotropic viruses from forest mosquitoes in eastern Columbia. J. Infect. Dis., 1944, **75:** 160–169.
161. Ross, R. W.: The Newala epidemic. III. The virus isolation, pathogenic properties and relation to the epidemic. J. Hyg., 1956, **54:** 177–191.
162. Rozeboom, L. E., and Burgdorfer, W.: Development of Colorado tick fever virus in the Rocky Mountain wood tick, *Dermacentor andersoni.* Amer. J. Hyg., 1959, **69:**138–145.
163. Ruesegger, J. M., Black, J., and Sharpless, G. R.: Primary antirabies immunization of man with HEP Flury virus vaccine. Amer. J. Pub. Health, 1961, **51:**706–716.
164. Sabin, A. B.: The dengue group of viruses and its family relationships. Bact. Rev., 1950, **14:**225–232.
165. Sabin, A. B.: Experimental studies on phlebotomus (pappataci, sandfly) fever during World War II. Arch. f. d. gesamte Virusf., 1951, **4:**367–410.
166. Sabin, A. B.: Research on dengue during World War II. Amer. J. Trop. Med. Hyg., 1952, **1:**30–50.
167. Sabin, A. B.: Recent advances in our knowledge of dengue and sandfly fever. Amer. J. Trop. Med. Hyg., 1955, **4:**198–207.
168. Sabin, A. B., and Schlesinger, R. W.: Production of immunity to dengue with virus modified by propagation in mice. Science, 1945, **101:**640–642.
169. Sanmartin-Barberi, C., Groot, H., and Osorno-Mesa, E.: Human epidemic in Columbia caused by Venezuelan equine encephalomyelitis virus. Amer. J. Trop. Med. Hyg., 1954, **3:**283–293.
170. Satriano, S. F., et al.: Investigations of eastern equine encephalomyelitis. IV. Susceptibility

and transmission studies with virus of pheasant origin. Amer. J. Hyg., 1958, **67:** 21–34.

171. Schaeffer, M., Kissling, R. E., and Chamberlain, R. W.: Current views in the North American arthropod-borne virus problem. Amer. J. Pub. Health, 1958, **48:**336–343.
172. Schaeffer, M., Robinson, R. Q., and Fodor, A. R.: New viruses and virus diseases of man. Ann. Rev. Microbiol., 1959, **13:**335–348.
173. Schindler, R.: Studies on the pathogenesis of rabies. Bull. W. H. O., 1961, **25:**119–126.
174. Schindler, R.: Protective effect of antirabies serum after intracerebral or intramuscular administration. Bull. W. H. O., 1961, **25:** 127–128.
175. Schlesinger, R. W., et al.: Clinical and serologic response of man to immunization with attenuated dengue and yellow fever viruses. J. Immunol., 1956, **77:**352–364.
176. Schmidt, J. R., Gajdusek, D. C., Schaeffer, M., and Gorrie, R. H.: Epidemic jungle fever among Okinawan colonists in the Bolivian rain forest. II. Isolation and characterization of Uruma virus, a newly recognized human pathogen. Amer. J. Trop. Med. Hyg., 1959, **8:**479–487.
177. Schmidt, J. R., Schmidt, M. L., and McWilliams, J. G.: Isolation of phlebotomus fever virus from *Phlebotomus papatasi.* Amer. J. Trop. Med. Hyg., 1960, **9:**450–454.
178. Schnurrenberger, P. R., Anderson, G. R., Russell, J. H., and Wentworth, F. H.: Avian embryo rabies immunization. II. A comparison of the antigenicity of high egg-passage and duck-embryo vaccines administered intradermally in man. Amer. J. Hyg., 1961, **74:**1–6.
179. Shah, K. V., et al.: Isolation of five strains of Sindbis virus in India. Indian J. Med. Res., 1960, **48:**300–308.
180. Silber, L. A., and Soloviev, V. D.: Far Eastern tick-borne spring-summer (spring) encephalitis. Amer. Rev. Soviet Med., 1946, Spec. Suppl., pp. 1–80.
181. Smadel, J. E.: Epidemic hemorrhagic fever. Amer. J. Pub. Health, 1953, **43:**1327–1330.
182. Smadel, J. E., et al.: Lymphocytic choriomeningitis: Two human fatalities following an unusual febrile illness. Proc. Soc. Exptl. Biol. Med., 1942, **49:**683–686.
183. Smithburn, K. C.: Studies on certain viruses isolated in the tropics of Africa and South America. Immunological reactions as determined by cross-neutralization tests. J. Immunol., 1952, **68:**441–460.
184. Smithburn, K. C.: Antigenic relationship among certain arthropod-borne viruses as revealed by neutralization tests. J. Immunol., 1954, **72:**376–388.
185. Smithburn, K. C., and Haddow, A. J.: Semliki forest virus. I. Isolation and pathogenic properties. J. Immunol., 1944, **49:**141–157.
186. Smithburn, K. C., and Haddow, A. J.: Ntaya virus. A hitherto unknown agent isolated from mosquitoes collected in Uganda. Proc. Soc. Exptl. Biol. Med., 1951, **77:**130–133.
187. Smithburn, K. C., Haddow, A. J., and Mahaffy, A. F.: A neurotropic virus isolated from Aedes mosquitoes caught in the Semliki forest. Amer. J. Trop. Med., 1946, **26:**189–208.
188. Smithburn, K. C., Kokernot, R. H., Weinbren, M. P., and De Meillon, B.: Isolation of Wesselsbron virus from a naturally infected human being and from *Aedes* (*Banksinella*) *circumluteolus* Theo. South African J. Med. Sci., 1957, **22:**113–120.
189. Smithburn, K. C., Mahaffy, A. F., and Paul, J. H.: Bwamba fever and its causative virus. Amer. J. Trop. Med., 1941, **21:**75–90.
190. Smorodintsev, A. A.: Tick-borne spring-summer encephalitis. Progress Med. Virol., 1958, **1:**210–248.
191. Southham, C. M., and Moore, A. E.: West Nile, Ilheus, and Bunyamwera virus infections in man. Amer. J. Trop. Med., 1951, **31:**724–741.
192. Stamm, D. D.: Studies on the ecology of equine encephalomyelitis. Amer. J. Pub. Health, 1958, **48:**328–335.
193. Steyn, J. J., and Schulz, K. H.: *Aedes* (*Ochlerotatus*) *caballus* Theobald, South African vector of Rift Valley fever. South African Med. J., 1955, **29:**1114–1120.
194. Strode, G. K. (ed.): Yellow Fever. McGraw-Hill, New York. 1951.
195. Sulkin, S. E.: Bat rabies: Experimental demonstration of the "reservoiring mechanism." Amer. J. Pub. Health, 1962, **52:**489–498.
196. Symposium: Symposium on epidemic hemorrhagic fever; Far East Command conference on hemorrhagic fever: Introduction. Ann. Intern. Med., 1953, **38:**53–60.
197. Symposium: Yellow fever in Africa. Bull. W. H. O., 1954, **11:**315–507.
198. Symposium: Symposium on the evolution of arborvirus diseases. Trans. Roy. Soc. Trop. Med. Hyg., 1960, **54:**89–134.
199. Symposium: Biology of viruses of the tick-borne encephalitis complex. Academic Press, New York. 1962.
200. Taylor, R. M., et al.: A newly recognized arthropod-transmitted virus. Amer. J. Trop. Med. Hyg., 1955, **4:**844–862.
201. Taylor, R. M., Work, T. H., Hurbut, H. S., and Rizk, F.: A study of the ecology of West Nile virus in Egypt. J. Trop. Med. Hyg., 1956, **5:**579–620.
202. Theiler, M.: Action of sodium desoxycholate on arthropod borne viruses. Proc. Soc. Exptl. Biol. Med., 1957, **96:**380–382.
203. Theiler, M., and Casals, J.: The serological reactions in yellow fever. Amer. J. Trop. Med. Hyg., 1958, **7:**585–594.
204. Thomas, L. A., and Eklund, C. M.: Overwintering of western equine encephalomyelitis virus in experimentally infected garter snakes and transmission to mosquitoes. Proc. Soc. Exptl. Biol. Med., 1960, **105:**52–55.
205. Thomas, L. A., and Eklund, C. M.: Use of the complement fixation test as a diagnostic aid in Colorado tick fever. J. Infect. Dis., 1960, **107:**235–240.
206. Thomas, L. A., and Eklund, C. M.: Overwintering of western equine encephalomyelitis virus in garter snakes experimentally infected by *Culex tarsalis.* Proc. Soc. Exptl. Biol. Med., 1962, **109:**421–424.
207. Thomas, L. A., Eklund, C. M., and Rush, W. A.: Susceptibility of garter snakes (Thamnophis spp) to Western equine encephalomyelitis virus. Proc. Soc. Exptl. Biol. Med., 1958, **99:**698–699.
208. Thomas, L. A., Kennedy, R. C., and Eklund, C. M.: Isolation of a virus closely related

to Powassan virus from *Dermacentor andersoni* collected along North Cache La Poudre River, Colorado. Proc. Soc. Exptl. Biol. Med., 1960, **104:**355–359.

209. Tierkel, E. S., and Neff, H. O.: Rabies: Methods in laboratory diagnosis. Public Health Service Publication No. 568. U. S. Department of Health, Education and Welfare. 1957.
210. Tiggertt, W. D., and Berge, T. O.: Japanese B encephalitis. Amer. J. Pub. Health, 1957, **47:**713–718.
211. Trapido, H., and Galindo, P.: Epidemiology of yellow fever in middle America. Exptl. Parasit., 1956, **5:**285–323.
212. Traub, E.: A filterable virus recovered from white mice. Science, 1935, **81:**298–299.
213. Varma, M. G. R., Webb, H. E., and Pavri, K. M.: Studies on the transmission of Kyasanur forest disease virus by *Haemaphysalis spinigera* Newman. Trans. Roy. Soc. Trop. Med. Hyg., 1960, **54:**509–516.
214. Veeraraghavan, N., and Subramanyan, T. P.: Antigenic value of lyophilized phenolized antirabies vaccine. Bull. W. H. O., 1961, **25:** 115–117.
215. Warren, J.: Epidemic encephalitis in Far East: Review. Amer. J. Trop. Med., 1946, **26:**417–436.
216. Warren, J., Smadel, J. E., and Russ, S. B.: The family relationship of encephalomyocarditis, Columbia SK, MM, and Mengo encephalomyelitis viruses. J. Immunol., 1949, **62:**387–398.
217. Weinbren, M. P., Heymann, C. S., Kokernot, R. H., and Paterson, H. E.: Simbu virus, a hitherto unknown agent isolated from *Aedes* (*Banksinella*) *circumluteolus.* South African J. Med. Sci., 1957, **22:**93–102.
218. Weinbren, M. P., Williams, M. C., and Haddow, A. J.: A variant of Rift Valley fever virus. South African Med. J., 1957, **31:**951–957.
219. Welsh, H. H., Neff, B. J., and Lennette, E. H.: The isolation and identification of Turlock virus in tissue culture. Amer. J. Trop. Med. Hyg., 1958, **7:**536–542.
220. Williams, M. C., and Woodall, J. P.: O'nyong-nyong fever: An epidemic virus disease in East Africa. II. Isolation and some properties of the virus. Trans. Roy. Soc. Trop. Med. Hyg.. 1961, **55:**135–141.
221. Wolman, M., and Behar, A.: Cytochemical study of nature of Negri bodies. J. Infect. Dis., 1952, **91:**69–71.
222. Woodruff, A. M.: The hemorrhagic fevers of Central and Eastern Asia. Bull. Hyg., 1953, **28:**233–236.
223. Work, T. H.: Virological aspects of Kyasanur forest disease. J. Indian Med. Assn., 1958, **31:**111–113.
224. Work, T. H.: Russian spring-summer virus in India. Kyasanur forest disease. Progress Med. Virol., 1958, **1:**248–277.
225. Work, T. H., Roderíguez, F. R., and Bhatt, P. N.: Virological epidemiology of the 1958 epidemic of Kyasanur forest disease. Amer. J. Pub. Health, 1959, **49:**869–874.
226. von Zeipel, G., Svedmyr, A., Holmgren, B., and Lindahl, J.: Tick-borne meningoencephalomyelitis in Sweden. Lancet, 1958, **i:** 104.

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